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RESEARCH



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INCIDENCE AND CHARACTERS OF VIBRIOS IN WATERS IN NORTHERN INDIA.

BY

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Cholera Inquiry under the Indian Research Fund Association.

(From the Central Research Institute, Kasauli.)

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THE present investigation was undertaken for the purpose of determining the incidence of vibrios in waters and the characters of strains in areas where cholera is not endemic, the object being to throw light on the significance of vibrios of types other than typical *V. cholerae* which are frequently isolated from human and other sources in endemic areas or in areas where cholera is epidemic.

The earlier observations on vibrios in water in India which have been recorded were based on investigations made before adequate methods of differentiating the characters of strains were available. Later work, most of which was carried out in cholera areas, showed that vibrios of different types could be isolated from water but the general distribution of the vibrio group in water has not been the subject of any extensive study. It will be of interest to summarize certain of the previous work on the subject in India and the conclusions which have been based on the observations made during successive phases of cholera investigations.

PREVIOUS INVESTIGATIONS ON VIBRIOS IN WATERS IN INDIA.

Hankin (1895) took up this subject for the purpose of obtaining information as to the nature and significance of vibrios found in water, certain evidence obtained elsewhere having led to conflicting opinions. He quotes Metchnikoff, Sanarelli, and others as having found, at places where no cholera existed at the time, vibrios in water which were practically indistinguishable from that of cholera. Large numbers of persons were seen to use such waters without developing cholera

and the discoverers came to the conclusion that there existed in nature a number of harmless vibrios which could not be distinguished from the cholera vibrio by the tests known at the time. It was therefore impossible to say whether water contained the microbes of cholera or merely a harmless vibrio showing similarity with it. The possibility that such strains were the true cholera vibrio in a state of degeneration had been suggested.

In this investigation the tests which Hankin used for the cholera vibrio were apparently the formation of a pellicle in peptone water containing comma-shaped bacilli and pathogenicity on injection into guinea-pigs. The tests for indol and liquefaction of gelatin were also in use at the time. He examined numerous waters in the United Provinces, especially at times of pilgrimages when cholera outbreaks occurred.

From wells and other water sources he isolated vibrios and found a much higher percentage to give positive results during and immediately after outbreaks of cholera than at other times. Hankin states that vibrios isolated when cholera was present were more highly pathogenic to guinea-pigs than those isolated under other circumstances. In summarizing his results he stated that microbes resembling that of cholera were rarely found except in places where cholera had recently existed, they were most virulent during an epidemic, and after the cessation of an epidemic they showed greatly diminished virulence for the guinea-pig. Those isolated after an epidemic also showed a diminished power of growing in agar and he took this as an indication of diminished vitality and virulence. His conclusion was that such vibrios were nothing more than the cholera vibrio in a degenerated condition.

Haffkine and Simpson (1895) took up similar lines of investigation in India at the same time and in their account of the work they summarized the experience of observers in Europe and elsewhere on vibrios in water. Many differences in strains had been noted and D. D. Cunningham is quoted as finding wide differences in comma bacilli isolated from cholera cases as well as from water.

Haffkine and Simpson carried out their work in Calcutta and its vicinity, the sources investigated being tanks, wells, drains, and the River Hooghly. Vibrios were isolated from 91 per cent of tanks in the vicinity of which cholera existed and from only 18 per cent of those in which it had not been present for a month or more. They correlated the incidence of cholera with the presence of vibrios in tanks and expressed the opinion that 'in nature there appear to be no water commas except those connected with cholera, varying, as we found them, in their morphological and physiological and biological characters'.

At a later stage of cholera research when serological methods of diagnosis were available Greig (1915) undertook a fresh investigation into vibrios in tanks and other open water sources in Calcutta. He did not record the proportion of samples from which vibrios were successfully isolated but carried out a detailed serological examination of 39 strains. He was at the time using agglutinating sera prepared against the whole vibrio and presumably containing both 'H' + 'O' agglutinins but, as noted by Gardner and Venkatraman (1935), the methods of test employed probably demonstrated the presence of different 'O' antigens. Greig succeeded in classifying the majority of his strains in six serological groups differing from that of *V. cholerae* and a smaller number of his strains remained

unclassified with the sera used. His water strains were not agglutinated by sera prepared against certain strains (other than typical *V. cholerae*) isolated from cholera cases. Greig (1914) records an instance of true *V. cholerae* being found in the protected reservoir of the Calcutta water-supply, this being traced to a man engaged in collecting water samples who was a carrier.

Gloster (1912) carried out an examination of water in Bombay City during a short outbreak of cholera and came to the conclusion that the vibrios he isolated were not modified *V. cholerae*.

Extensive work on this subject was carried out by Tomb and Maitra (1926, 1927) during the course of an investigation into cholera in the Asansol Mining Settlement of Bengal. In this area tanks were extensively used for water-supply and they were frequently used for washing after defaecation. During the cold weather, when cholera was absent from the Settlement, vibrios were found to be practically absent from the tanks but with the onset of cholera in March at the beginning of the hot weather inagglutinable vibrios appeared in all tanks. These disappeared when the cholera epidemic subsided. When washing in a tank was prohibited the vibrios diminished in number and they disappeared in 12 to 14 days. On three occasions only out of a very large number of examinations of tank water was the agglutinable vibrio isolated. In those cases contamination with cholera dejecta had occurred within 24 hours.

Tomb and Maitra infected the water of a tank with cholera stools containing the agglutinable vibrio and after 12 to 14 hours recovered only inagglutinable strains. When cholera cultures were added instead of infected faeces recovery of the agglutinable strain was not made after 36 hours. They concluded that the vibrio had changed from the agglutinable to the inagglutinable form. Tomb and Maitra also found that, while cholera convalescents did not discharge agglutinable vibrios for more than two to four weeks, 30 per cent of them became 'chronic carriers' of inagglutinable vibrios. On the basis of these observations on water and carriers they related the endemicity of cholera to the presence of carriers of inagglutinable strains.

d'Herelle and Malone (1930) made a series of observations on the presence of vibrios in wells in the United Provinces and the Punjab. They found that vibrios were absent from wells in areas which had been free from cholera for a prolonged period. Where cholera had been absent for two years a small proportion of wells contained inagglutinable vibrios. In places in which cholera had recently occurred inagglutinable vibrios were found in a large proportion of well waters.

Pasricha (1931) recorded the results of the examination of waters in Bengal where inagglutinable vibrios had been isolated from 59 out of 306 water samples. He found the incidence of vibrios to follow closely the incidence of cholera, these being most prevalent at the beginning of the cholera season.

Saranjam Khan (1930) in the course of an investigation into the epidemiology of cholera in the United Provinces, where the disease is not endemic but where epidemics frequently occur on the introduction of infection from other areas, noted the presence of inagglutinable vibrios in numerous water sources. He did not record their percentage incidence or investigate their characters in detail, but as the result of his epidemiological observations he excluded the water vibrios from taking any part in the causation of cholera.

PRESENT INQUIRY.

In the course of an investigation into the characters of vibrios isolated from cases of clinical cholera, carriers, and water, the results of which have been reported by Taylor, Pandit and Read (1937), the methods employed had enabled certain classifications to be made on the basis of 'O' serology and biochemical reactions. Numerous different 'O' serological types had been defined and a proportion of water vibrios had been placed in special serological groups. Vibrios from water had in some instances been found to be of the same types as strains other than typical *V. cholerae* of 'O' group I (Gardner and Venkatraman, *loc. cit.*) isolated from cases of clinical cholera or from healthy individuals. Amongst the strains examined was a series isolated from open water sources in Rajputana where cholera is not endemic and outbreaks seldom occur. Out of 83 sources examined 59 yielded vibrios and some of them were agglutinated with the same special 'O' sera as strains from clinical cholera cases in Bengal. This incidence of vibrios was very much higher than had previously been found under similar circumstances in India and the finding that strains isolated appeared to be of similar type as those obtained from cholera cases suggested the advisability of determining further the distribution of such strains in natural waters and especially their incidence under conditions where cholera contamination could be excluded.

TECHNIQUE.

The method of examination employed consisted of enrichment of large quantities of water with concentrated alkaline peptone solution followed by plating on Aronson's medium. Enrichment was carried out in 10-ounce screw-capped medical flats (United Glass Bottles Manufacturers, Ltd.). Two hundred c.c. of the water sample under examination was collected in the bottle and 20 c.c. of 10 per cent peptone and 5 per cent sodium chloride added. The reaction was raised to pH 9.0 by the addition of N/1 NaOH. As a rule 1.0 c.c. to 1.5 c.c. was required; thymol blue was used as the indicator.

The procedure subsequently followed depended on the circumstances of collection. When collection was carried out by a member of the Institute staff during a tour for the purpose the bottle was kept overnight at room temperature which, at the time of the year the work was carried out, resulted in adequate growth, and in the morning 2 c.c. was transferred to a sealed ampoule and despatched by post to the Institute. If the officer was returning to Kasauli on the day of collection the bottle was taken direct to Kasauli. In the case of samples collected for us by other laboratories at a distance enrichment was carried out by them and capsules of the enriched material sent. Public Health Officers in the Punjab and United Provinces were supplied with sterile screw-capped bottles containing 20 c.c. of the concentrated alkaline peptone solution and after adding 200 c.c. of water to each bottle they despatched the whole bottle to the Institute. From material received under these conditions 2 c.c. was added to 10 c.c. of peptone water and after 6 hours' incubation 1 drop was plated on Aronson's medium. Our experience of this medium has been highly satisfactory. It has been found to inhibit satisfactorily the growth of *coli*-group organisms and *proteus*, while yielding a growth of vibrios which is little less than that obtained on agar, provided the medium is used immediately after preparation. At the full strength of alkalinity recommended

in the original description of the medium there is some degree of restriction of growth of vibrios but if the sodium carbonate used to produce a high degree of alkalinity is reduced to five-sixths of that recommended, there is little reduction in the growth of vibrios, while *coli*-group organisms are inhibited and few, if any, colonies of *proteus* will develop and these will not be of the spreading type. Organisms of the *aerogenes*-group will grow if the inoculum is heavy.

OBSERVATIONS DURING THE HOT-WEATHER AND MONSOON PERIODS.

The areas selected for the examination of waters were the districts of Ambala, Karnal, and Multan, in the Punjab, and the vicinity of Lucknow in the United Provinces.

Cholera is not endemic in the Punjab and although it occurs in some years in epidemic form the areas affected are usually limited and can be defined in any one year. For example, in the Karnal district the total deaths from cholera which were recorded in 1936 were 7, and in 1937 (the year in which investigations were carried out) the total was 9 deaths. The corresponding figures for Ambala district were 6 and 54. It was thus possible on the known distribution of the cases to select places for the examination of waters in which a likelihood of contamination from cholera cases was very remote. In the United Provinces cholera is also not endemic but on introduction a high epidemicity frequently occurs. Places could, however, be selected in these Provinces where the chance of infection of water during the previous year was unlikely.

The sources examined were shallow wells, tanks (excavated areas containing permanent collections of water used for drinking or washing), and rivers.

A series of visits was made to the selected areas and the results of the examination of each group of water samples collected are given below:—

Punjab water samples—Series 1.

Nineteen water sources were examined on 24th and 25th April, 1937, during the hot-weather period, in the vicinity of villages in which cholera had not occurred during the previous year. Vibrios were isolated from all the sources and the details of strains, one each of any biochemical or serological type from each source, are given in Table I. Vibrios of more than one type were isolated from the majority of samples and 35 strains were finally selected for detailed examination of their characters.

The incidence of the different Heiberg fermentation types was:—

Type I	Type II	Type III	Type IV	Type V	Type VI
8	18	0	7	0	2

No strain was agglutinable with pure 'O' cholera serum of the Inaba or Ogawa type. Twelve strains agglutinated to 50 per cent or over with Inaba 'H' + 'O' serum and two strains to 25 per cent.

All strains were examined with the special 'O' sera prepared against vibrios selected from inagglutinable strains derived from cases of clinical cholera, carriers, and water, the details of which have been given in a previous communication

(Taylor, Pandit and Read, *loc. cit.*) and 7 strains were found to agglutinate with one or other of the sera. This point is discussed in summarizing the results.

Punjab water samples—Series 2.

A second series of samples was examined in Ambala and Karnal districts from 15th to 17th July, 1937, representing hot-weather conditions. In this series some of the same samples as were examined in the first series were re-examined and a total of 21 sources was dealt with. Again vibrios were obtained from all sources. The majority of sources showed more than one type of vibrio and a total of 38 strains was selected for examination the details of which are given in Table II.

The vibrios isolated belonged to the following Heiberg types:—

Type I	Type II	Type III	Type IV	Type V	Type VI
8	14	3	3	0	10

No strain agglutinated with pure 'O' group I serum (Inaba or Ogawa type), while 12 strains agglutinated with Inaba 'H' + 'O' serum.

Thirteen strains showed agglutination with the special test sera.

Punjab water samples—Series 3.

Nineteen samples from the Multan and Ambala districts were collected and enriched for us by the Public Health staff, Punjab Government, during August 1937, representing monsoon conditions. Vibrios were isolated from all samples. From these 24 strains were selected for examination the details of which are given in Table III.

The following was the distribution of Heiberg types in this group:—

Type I	Type II	Type III	Type IV	Type V	Type VI
8	10	2	0	0	4

None of the strains agglutinated with 'O' group I serum, while 7 agglutinated with Inaba 'H' + 'O' serum.

Five strains showed agglutination with the special test sera.

Punjab water samples—Series 4.

In connection with certain other observations two shallow wells in the village of Jhanjhari, Karnal district, were examined in August 1937. Both wells yielded vibrios and 7 strains were isolated (Table IV). Of these four belonged to Heiberg type I and three to Heiberg type II. From each of the wells a Heiberg type II strain was isolated which was of the same biochemical type, but serological identity of the strains was not established. The remaining five strains differed in biochemical and serological characters. Two of the strains agglutinated to 100 per cent of titre with Inaba 'H' + 'O' serum and four agglutinated to from 50 to 100 per cent of titre with sera prepared against Calcutta case, carrier, or water strains.

United Provinces—Lucknow series.

Thirteen samples were examined in Lucknow City and vicinity on 21st and 22nd May, 1937, during the hot weather when maximum temperatures up to 107°F. were being experienced. These included shallow wells, tanks, and river water. All sources were positive, while two control sources consisting of the filtered and chlorinated Goomti water and water from a tube-well did not show vibrios. Twenty-six strains of different biochemical or serological type were examined, details of which are given in Table V. The distribution of Heiberg types in this series was as follows:—

Type I	Type II	Type III	Type IV	Type V	Type VI
11	14	0	0	1	0

No strain agglutinated with 'O' group I serum (Inaba or Ogawa type), while 14 agglutinated to 50 per cent or over with Inaba 'H' + 'O' serum. Four agglutinated to varying percentages of titre with the special test sera.

United Provinces—Hardwar series.

The conditions under which the examination of water for vibrios at Hardwar was carried out differed from those of the other Punjab and United Provinces series. Many thousands of pilgrims from all over India visit Hardwar annually and bathe in the Ganges as part of their religious ceremonies. There is thus always a possibility of contamination of the water of the bathing ghat from cholera sources and during the period in which the samples were taken 94 cases of cholera occurred at Hardwar. Weekly samples were collected from the bathing ghat by the local public health staff during June, July, and August, a total of 11 enriched samples being received for examination. All samples were positive and 10 showed more than one type of vibrio. In Table VI details are given of the 30 strains whose characters were determined. The distribution of Heiberg types was as follows:—

Type I	Type II	Type III	Type IV	Type V	Type VI
9	9	1	5	2	4

In accordance with differences shown in the fermentation reactions, indol production, cholera red reaction, production of hæmolysin, and serological reactions, these have been placed in 15 groups (Table VII). As the 'O' serological type of many of the strains was not determined it is possible that there may be an even greater number of different types in the series. Out of the 30 strains 14 agglutinated with Inaba 'H' + 'O' serum and none with pure 'O' serum.

OBSERVATIONS DURING THE COLD-WEATHER PERIOD.

Examinations of wells and tanks were repeated in the Karnal and Ambala districts during the month of January 1938 certain of the same sources as had previously been examined again being tested. At the time of taking samples the maximum and minimum temperatures were in the vicinity of 70°F. and 40°F., respectively. Certain changes had in the interval taken place in local conditions,

some of the wells previously examined having been improved by lining them with cement and constructing plinths and high copings.

Seventeen water sources were examined the details of which are given in Table VIII. From 16 of these vibrios were isolated and from 9 more than one biochemical type were obtained.

A total of 123 colonies was examined and taking a single strain of each biochemical type isolated from any one sample 32 strains were retained for detailed examination.

Vibrios of all six Heiberg types were obtained but the proportions differed in some degree from those in the series isolated during the hot-weather and monsoon periods. There was a larger proportion of types V and VI. The distribution was as follows :--

Type I	Type II	Type III	Type IV	Type V	Type VI
9	2	1	4	7	9

None of the strains agglutinated 'O' group I serum or 'H' + 'O' serum. Six were found to agglutinate with special 'O' sera raised against case and carrier strains from different parts of India.

The results of this series of examination are summarized in Table X.

COMMENTS.

For the purpose of analysis of results the waters examined may conveniently be divided into three groups, viz., (a) the hot-weather and monsoon series collected under conditions in which the likelihood of cholera contamination could be excluded, (b) the cold-weather series under the same conditions, and (c) the Hardwar series.

The findings in the hot-weather and monsoon series are summarized in Table IX. All 74 sources examined yielded vibrios, which were of numerous different biochemical and serological types. The majority of strains were of Heiberg types I and II, and type VI strains constituted only 12 per cent of the total. No strain agglutinated with pure 'O' cholera serum, but 36 per cent agglutinated to 50 per cent or over with Inaba 'H' + 'O' serum. Fifteen per cent of strains agglutinated with 'O' sera prepared against vibrios, other than of 'O' group I, isolated from cases of clinical cholera and 8 per cent agglutinated with carrier strain sera.

During the cold weather vibrios were isolated from 16 out of 17 sources and the results of the examinations, as given in Table X, show certain differences from those of the hot-weather and monsoon series from the same area. The majority of strains belonged to Heiberg types V and VI although all six types were represented. None of the strains in this series were 'H' agglutinators but vibrios agglutinating with special 'O' sera prepared against case and carrier strains were included.

Although the Hardwar series were collected under conditions in which contamination from cholera sources might be possible the general characters of the strains, as indicated in Table XI, were very similar to those of the other samples

examined during hot-weather and monsoon periods. The distribution of Heiberg types was comparable. A higher incidence of 'H' agglutinators was noted, these being 46 per cent as compared with 36 per cent, but the difference is not significant in relation to the number of strains examined. The proportion of strains agglutinating with special 'O' case sera was lower than that found in the hot-weather and monsoon series collected in non-cholera areas.

The results of these examinations show that vibrios are almost universally present in unprotected wells, tanks, and rivers in areas of Northern India where cholera is not endemic, and that their presence is in no way related to contamination from cholera sources. From individual sources vibrios of several different types may be isolated and the vibrio group in water is extremely heterogeneous. A considerable proportion of water strains possesses an 'H' antigen in common with *V. cholerae* but no vibrio was isolated which showed 'O' serological relationship with that organism. Many of the strains isolated showed 'O' serological relationship with vibrios other than typical *V. cholerae* isolated from cases of clinical cholera and from carriers in Bengal and other parts of India (Tables XII and XIII).

It is obvious that with this almost universal distribution of vibrios in water, including waters which may be used for drinking purposes, vibrios must frequently obtain entrance to the intestinal tract of those using the water and the appearance of such vibrios in stools of healthy individuals or in cholera cases is to be expected. In the area in which the examination of waters was carried out 30 per cent of stools of a small group of individuals examined yielded vibrios.

The results of these examinations indicate a probable source of origin for the vibrios inagglutinable with 'O' group I serum which are not infrequently isolated from stools of cholera cases. Our findings add further evidence in support of the conclusion of Taylor, Pandit and Read (*loc. cit.*) that vibrios other than typical *V. cholerae*, as defined by its characteristic 'O' antigen, including strains which agglutinate with cholera 'H' + 'O' serum only, do not play a part in the aetiology of cholera.

SUMMARY.

1. Vibrios were found to be universally present in waters in Northern India and were isolated from 90 out of 91 samples of well, tank, and river waters under conditions in which cholera contamination could be excluded.

2. The vibrios were of very heterogeneous types as determined by their biochemical and serological reactions.

3. No strain was isolated which agglutinated with pure 'O' serum of 'O' group I. Out of 130 strains isolated during the hot-weather and monsoon periods 36 per cent agglutinated with 'H' + 'O' high-titre cholera serum.

4. 'O' serological relationship with vibrio strains isolated from cases of clinical cholera in different parts of India was shown in 14 per cent of strains examined.

5. A probable water source of origin of the vibrios, other than typical *V. cholerae*, which are sometimes found in stools of cholera cases, is indicated by these findings.

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TABLE I.

Punjab water samples—Series 1.

Strain number.	Source.	pH.	Chlorine parts per 100,000.	Colony characters.	BIOCHEMICAL REACTIONS.				SEROLOGICAL REACTIONS.	
					Hei-berg type.	Cholera red.	Indol.	Hemolysis.	V. P.	
W.A./1A	Kalka spring water, drinking supply	7.8	2.25	S. T.	II	+	+	N. H.	+	Inaba 'H' + 'O' serum.
W.A./2	Pinjaur tank water, drinking supply	7.4	4.0	S. O.	IV	—	+	H.	—	50 per cent
W.A./3A	Mubarakpur shallow well, drinking supply.	7.5	1.5	R. O.	II	—	—	N. H.	—	2898—25 per cent.
W.A./4A	Lalru tank water, not used for drinking.	0.5	1.0	R. O.	II	—	—	N. H.	—	Unclassified.
W.A./5	Ambala City tank water, not used for drinking.	7.8	3.4	S. T.	II	—	—	H.	+	"
W.A./6	Karnal lake water (Puran jhil) ..	7.2	2.0	S. O.	IV	—	+	H.	—	"
W.A./7A	Karnal well water, drinking supply	7.5	3.5	S. T.	IV	—	+	H.	—	"

S. T. = Smooth translucent; S. O. = Smooth opaque; R. O. = Rough opaque; H. = Hemolytic; N. H. = Non-hemolytic. All strains were Gram negative, monoflagellate, actively motile vibrios and inagglutinable with 'O' group I serum. The 'O' serological groups are those described by Taylor, Pandit and Read (*loc. cit.*). Strains agglutinating to less than 25 per cent of titre have not been taken as 'agglutinable' for the purpose of analysis of results.

TABLE I—*concl'd.*

Strain number.	Source.	pH.	Chlorine parts per 100,000.	Colony characters.	BIOCHEMICAL REACTIONS.				SEROLOGICAL REACTIONS.	
					Heiberg type.	Cholera red.	Indol.	Hæmolysis.	Inaba 'H' + 'O' serum.	'O' serological group.
W.A./8	Karnal tank water ..	7.8	2.0	R. O.	IV	—	+	H.	..	Unclassified.
W.A./9A	Karnal shallow well (Dabri), drinking supply.	7.6	27.5	S. O.	I	—	—	N. H.	..	"
W.A./9B	Do.	7.6	27.5	S. O.	I	—	—	H.	..	"
W.A./9D	Do.	7.6	27.5	R. O.	II	+	+	N. H.	50 per cent	"
W.A./10A	Karnal shallow well, drinking supply	7.6	11.5	S. O.	I	—	—	H.	50 "	"
W.A./10B	Do.	7.6	11.5	S. T.	II	+	+	H.	50 "	"
W.A./10C	Do.	7.6	11.5	S. T.	I	—	—	N. H.
W.A./10D	Do.	7.6	11.5	S. O.	II	+	+	N. H.	50 per cent	1805/1—50 per cent.
W.A./12A	Karnal shallow well (Gharonda), drinking supply.	8.0	22.5	S. T.	I	—	—	N. H.	..	Unclassified.
W.A./12B	Do.	8.0	22.5	S. T.	II	+	+	N. H.	..	"
W.A./12C	Do.	8.0	22.5	S. O.	I	+	+	N. H.	..	"
W.A./13A	Panipat tank water, drinking supply	7.6	1.0	S. O.	II	+	+	N. H.	50 per cent	"
W.A./13B	Do.	7.6	1.0	S. O.	II	+	+	N. H.	..	G. II—50 per cent.

W.A./14A	Panipat shallow well, not used for drinking.	7.8	73.0	S. O.	I	—	—	H.	—	..	8314—50 per cent.
W.A./14B	Do.	7.8	75.0	S. O.	I	—	—	N. H.	—	..	Unclassified.
W.A./14C	Do.	7.8	75.0	S. O.	II	—	—	N. H.	—	..	"
W.A./15A	Sonipat tank water, drinking supply	7.8	1.0	S. T.	II	+	+	N. H.	+	50 per cent	"
W.A./15B	Do.	7.8	1.0	S. O.	IV	—	+	H.	+	..	"
W.A./16	Shallow well (Delhi), used for drinking.	7.1	27.0	S. T.	II	+	+	N. H.	+	50 per cent	"
W.A./16D ₁	Do.	7.4	27.0	S. O.	II	+	+	N. H.	+	100 "	1612—25 per cent.
W.A./16D ₂	Do.	7.4	27.0	S. O.	IV	—	+	N. H.	+	..	Unclassified.
W.A./17A	River water Jumna Canal, used for drinking.	7.6	1.0	S. T.	II	+	+	N. H.	+	50 per cent	324/7—50 per cent.
W.A./17B	Do.	7.6	1.0	S. T.	II	—	—	N. II.	—	50 "	Unclassified.
W.A./17J ₁	Do.	7.6	1.0	S. O.	VI (1)	—	—	N. H.	—	..	"
W.A./18A	Tank water (Kurukshetra), drinking supply.	7.6	1.0	S. O.	II	—	—	N. H.	+	..	"
W.A./18B	Do.	7.6	1.0	S. O.	IV	—	+	H.	—	..	"
W.A./18K ₂	Do.	7.6	1.0	R. O.	VI	—	—	N. H.	—	..	"
W.A./19	Tank water (Samtirath), drinking supply.	S. T.	II	+	+	N. H.	+	50 per cent	G. II—25 per cent.

S. T. = Smooth translucent; S. O. = Smooth opaque; R. O. = Rough opaque; H. = Haemolytic; N. II. = Non-haemolytic. All strains were Gram negative, monoflagellate, actively motile vibrios and inagglutinable with 'O' group I serum. The 'O' serological groups are those described by Taylor, Pandit and Read (*loc. cit.*). Strains agglutinating to less than 25 per cent of titre have not been taken as 'agglutinable' for the purpose of analysis of results.

TABLE II.

Punjab water samples—Series 2.

Strain number.	Source.	pH.	Chlorine parts per 100,000.	Colony characters.	BIOCHEMICAL REACTIONS.					SEROLOGICAL REACTIONS.	
					Heiberg type.	Cholera red.	Indol.	Hæmolysis.	V. P.	'H' + 'O' serum.	'O' serological group.
A.J./1A	Tank water (Pinjaur), drinking supply.	7.0	2.0	S. O.	IV	—	+	N. H.	—	..	Unclassified.
A.J./1B	Do.	7.0	2.0	S. T.	VI (1)	—	—	N. H.	—	..	"
A.J./2B	Shallow well (Panchkoola), drinking supply.	7.4	2.0	S. O.	IV	—	+	H.	—	..	630/3T—50 per cent.
A.J./2C	Do.	7.4	2.0	S. O.	VI (1)	—	—	N. H.	—	..	Unclassified.
A.J./3	Shallow well (Mubarakpur), drinking supply.	7.4	3.0	S. T.	VI (1)	—	—	N. H.	—	..	"
A.J./4A	Tank water (Lalru), not used for drinking.	7.4	3.0	S. T.	II	+	+	N. H.	+	50 per cent	"
A.J./4B	Do.	7.4	3.0	S. O.	III	—	—	N. H.	—	..	3067—100 per cent.
A.J./4C	Do.	7.4	3.0	S. O.	VI (1)	—	—	N. H.	—	..	Unclassified.
A.J./4D	Do.	7.4	3.0	S. O.	III	—	—	H.	—	..	L/1/36—100 per cent.
A.J./5A	Tank water (Ambala City), used for drinking.	7.6	2.0	S. O.	II	—	+	H.	+	50 per cent	Unclassified
A.J./5B	Do.	7.6	2.0	S. O.	VI (1)	—	—	N. H.	+	..	"
A.J./5C	Do.	7.6	2.0	S. O.	II	+	+	N. H.	+	50 per cent	"

A.J./6B	Well water (Ambala City), used for drinking.	7-6	2-0	R. O.	VI (1)	-	-	N. H.	+	..	"
A.J./7A	Shallow well (Shahabad), drinking supply.	7-4	5-0	S. O.	VI (1)	-	-	N. H.	-	..	"
A.J./7B	Do.	7-4	5-0	S. O.	VI (2)	-	-	N. H.	+	..	"
A.J./8A	Tank water (Kurukshetra), drinking supply.	7-0	2-0	S. O.	I	+	+	N. H.	+	50 per cent	L/9/36-100 per cent.
A.J./8B	Do.	7-0	2-0	S. O.	II	+	+	N. H.	+	100	Unclassified.
A.J./9A	Shallow well (Thanesar), drinking supply.	7-6	11-5	S. O.	VI (1)	-	-	N. H.	+	..	"
A.J./9B	Do.	7-6	11-5	S. O.	VI (2)	-	-	N. H.	-	..	"
A.J./10	Tank water (Karnal), used for drinking.	7-8	1-5	S. O.	I (a)	+	+	N. H.	-	..	"
A.J./11A	Shallow well (Majoorā village), drinking supply.	7-6	7-5	S. T.	I	-	-	P. H.	+	..	"
A.J./11B	Do.	7-6	7-5	S. O.	II	+	+	N. H.	+	50 per cent	324/7-25 per cent.
A.J./12A	Do.	7-6	7-5	S. O.	II	-	-	N. H.	+	50	G. III-50 per cent.
A.J./12B	Do.	7-6	7-5	S. O.	II	-	-	N. H.	-	50	984-25 per cent.
A.J./12C	Do.	7-6	7-5	S. O.	II	+	+	N. H.	+	50	Unclassified.
A.J./12D	Do.	7-6	7-5	S. O.	II	-	-	N. H.	-	50	5780-50 per cent.
A.J./13B	Shallow well (Karnal City), drinking supply.	7-4	5-0	S. O.	I	+	+	N. H.	+	50	8314-25 per cent.

S. T. = Smooth translucent; S. O. = Smooth opaque; R. O. = Rough opaque; H. = Haemolytic; N. H. = Non-haemolytic; P. H. = Partially haemolytic. All strains were Gram negative, monoflagellate, actively motile vibrios and inagglutinable with 'O' group I serum. The 'O' serological groups are those described by Taylor, Pandit and Read (*loc. cit.*). Strains agglutinating to less than 25 per cent of titre have not been taken as 'agglutinable' for the purpose of analysis of results.

TABLE II—*concl'd.*

Strain number.	Source.	pH.	Chlorine parts per 100,000.	Colony characters.	BIOCHEMICAL REACTIONS.				SEROLOGICAL REACTIONS.	
					Hei-berg type.	Cholera red.	Indol.	Hæm-olysis.	'H' + 'O' serum.	'O' serological group.
A.J./14A	Shallow well (Karnal City), drinking supply.	7.4	3.0	S. T.	II	+	+	N. H.	..	G. VI—100 per cent. Unclassified.
A.J./14B	Do.	7.4	3.0	S. T.	III	—	—	N. H.
A.J./15A	Shallow well (Jhanjhari village), drinking supply for Hindus.	7.4	27.5	S. O.	II	+	+	N. H.
A.J./15B	Do.	7.4	27.5	S. O.	I	+	+	N. H.	..	8314—25 per cent. Unclassified.
A.J./16	Shallow well (Jhanjhari), drinking supply for Chamars.	7.6	15.0	S. O.	I	—	—	N. H.
A.J./17	Shallow well (Budhakhara), used for drinking.	7.4	5.0	S. T.	II	+	+	N. H.
A.J./18A	Shallow well (Panipat City), not used for drinking.	7.4	90.0	S. O.	I	+	+	N. H.	..	L/9/36—100 per cent. Unclassified.
A.J./18B	Do.	7.4	90.0	S. O.	I	+	+	N. H.
A.J./19A	Shallow well (Sonipat City), used for drinking.	7.8	9.0	S. O.	II	+	+	N. H.	100 per cent	..
A.J./20A	Shallow well (Delhi), used for drinking.	7.8	10.0	S. O.	IV	—	+	H.
A.J./21A	Stagnant pond (Delhi), not used for drinking.	..	17.5	S. O.	II	+	+	N. H.

S. T. = Smooth translucent; S. O. = Smooth opaque; R. O. = Rough opaque; H. = Hæmolytic; N. H. = Non-hæmolytic; P. H. = Partially hæmolytic. All strains were Gram negative, monoflagellate, actively motile vibrios and inagglutinable with 'O' group I serum. The 'O' serological groups are those described by Taylor, Pandit and Read (*loc. cit.*). Strains agglutinating to less than 25 per cent of titre have not been taken as 'agglutinable' for the purpose of analysis of results.

TABLE III.
Punjab water samples—Series 3.

Strain number.	Source.	Colony characters.	BIOCHEMICAL REACTIONS.				SEROLOGICAL REACTIONS.	
			Heiберг type.	Cholera red.	Indol.	Hæmoly- sis.	V. P.	'H' + 'O' serum.
L./1	Shallow well (Dhulkot village), drinking supply	S. O.	II	+	+	P. H.	+	100 per cent
L./2(2)A	Shallow well (Babyal village), drinking supply	S. T.	I	+	+	N. H.	+	..
L./2(2)O	Do.	S. O.	I	+	+	N. H.	+	..
L./2(2)B	Do.	S. T.	VI (1)	—	—	N. H.	—	..
L./3	Shallow well (Mandour village), drinking supply.	S. T.	II	—	—	N. H.	—	..
L./5A	Shallow well (Khatali village), drinking supply.	S. T.	I	+	+	N. H.	+	100 per cent
L./5B	Do.	S. O.	II	+	+	N. H.	+	100 "
L./6	Do.	S. T.	III	—	+	H.	—	..
L./7	Shallow well (Garewala village), drinking supply.	S. T.	VI (2)	—	—	N. H.	—	..
L./8	Shallow well (Barwala village), drinking supply	S. T.	I	—	—	H.	+	..
L./9	Shallow well (Sultanpur village), drinking supply.	S. T.	II	—	—	N. H.	—	..
L./10	Shallow well (Knkru village), drinking supply.	S. T.	II	—	—	N. H.	—	..
								5780—100 per cent.

S. T. = Smooth translucent; S. O. = Smooth opaque; H. = Hemolytic; N. H. = Non-hemolytic; P. H. = Partially hemolytic. All strains were Gram negative, monellagellate, actively motile vibrios and inagglutinable with 'O' group I serum. The 'O' serological groups are those described by Taylor, Pandit and Read (*loc. cit.*). Strains agglutinating to less than 25 per cent of titre have not been taken as 'agglutinable' for the purpose of analysis of results.

TABLE III—*concl'd.*

Strain number.	Source.	Colony characters.	BIOCHEMICAL REACTIONS.				SEROLOGICAL REACTIONS.	
			Hei- berg's type.	Cholera red.	Indol.	Hæmoly- sis.	V. P.	Inaba 'H' + 'O', serum.
L./11	Shallow well (Multan City)	..	III	—	—	N. H.	—	..
L./12(1)	Shallow well (Chawring, Multan district)	..	I	+	+	N. H.	—	..
L./13(3)	Shallow well (Kasab, Multan district)	..	II	—	—	N. H.	—	..
L./14A	Shallow well (Dabkar, Multan district)	..	I	+	+	P. H.	+	50 per cent
L./14B	Do.	..	II	+	+	N. H.	+	..
L./14C	Do.	..	I	+	+	N. H.	+	50 per cent
L./15(2)	Shallow well (Basti Paran, Multan district)	..	VI	—	—	N. H.	—	..
L./16(1)	Shallow well (Veelgunj, Multan district)	..	II	+	+	N. H.	—	50 per cent
L./17(1)	Shallow well (Mohalla Sultani, Multan City)	..	I	+	+	N. H.	+	50
L./18(1)	Shallow well (Mohalla Sutribut, Multan City)	..	II	—	—	N. H.	—	..
L./19B	Shallow well (Toi Alam Shah, Multan)	..	II	—	—	N. H.	—	..
L./20(1)	Shallow well (Pathanan, Multan)	..	VI	—	—	N. H.	—	..
								L/9/36—25 per cent.

S. T. = Smooth translucent; S. O. = Smooth opaque; H. = Hamolytic; N. H. = Non-hamolytic; P. H. = Partially hæmolytic. All strains were Gram negative, monoflagellate, actively motile vibrios and inagglutinable with 'O' group I serum. The 'O' serological groups are those described by Taylor, Pandit and Read (*loc. cit.*). Strains agglutinating to less than 25 per cent of titre have not been taken as 'agglutinable' for the purpose of analysis of results.

TABLE IV.

Punjab water samples—Series 4.

Strain number.	Source.	Colony characters.	BIOCHEMICAL REACTIONS.				SEROLOGICAL REACTIONS.		
			Heiberg type.	Cholera red.	Indol.	Hæmolysis.	V. P.	Inaba 'H' + 'O' serum.	'O' serological group.
W.Jh./2A	Shallow well } for Hindus. }	S. O.	I	+	+	N. H.	+	100 per cent	1805/1—50 per cent.
W.Jh./2B	Do.	S. O.	II	+	+	N. H.	+	..	Unclassified.
W.Jh./2C	Do.	S. O.	I	+	+	N. H.	—	..	8314—100 per cent.
W.Jh./2E	Do.	S. O.	I	+	+	N. H.	+	..	Unclassified.
W.Jh./3A	Shallow well } for Chamars. }	S. O.	II	+	+	N. H.	+
W.Jh./3B	Do.	S. O.	II	+	+	N. H.	+	100 per cent	2898—50 per cent.
W.Jh./3C	Do.	S. O.	I	+	+	N. H.	+	..	Tank 4B—50 per cent.

S. O. = Smooth opaque; N. H. = Non-hæmolytic. All strains were Gram negative, nonflagellate, actively motile vibrios and inagglutinable with 'O' group I serum. The 'O' serological groups are those described by Taylor, Pandit and Read (*loc. cit.*). Strains agglutinating to less than 25 per cent of titre have not been taken as 'agglutinable' for the purpose of analysis of results.

TABLE V.

United Provinces—Lucknow series.

Strain number.	Source.	pH.	Chlorine parts per 100,000.	Colony characters.	BIOCHEMICAL REACTIONS.					SEROLOGICAL REACTIONS.	
					Hei-berg type.	Cholera red.	Indol.	Hæmolysis.	V. P.	Inaba 'H' + 'O' serum.	'O' serological group.
A.M./1	Well water (Railway Station, Lucknow).	7.6	11.0	S. T.	I	—	—	P. H.	+	..	Unclassified.
A.M./2A	Raw unfiltered water (Gomti river)	7.8	2.0	S. O.	II	+	+	N. H.	+	50 per cent	"
A.M./2B	Do.	7.8	2.0	S. T.	I	—	—	N. H.	+	..	"
A.M./3	Same as A.M./2A after slow sand filtration.	7.8	2.0	S. O.	II	+	+	N. H.	+	50 per cent	"
A.M./4A	Shallow well water (Ashbagh, Sweeper Colony), drinking supply.	7.6	11.5	S. O.	I	+	+	P. H.	+	100	"
A.M./4B	Do.	7.6	11.5	S. O.	I	+	+	N. H.	+	100	"
A.M./5	Shallow well water (Lucknow City), not used for drinking.	7.3	11.0	S. T.	II	+	+	N. H.	+	100	"
A.M./6A	Shallow well near drain, drinking supply.	7.3	65.0	S. O.	I	+	+	N. H.	—	100	29.43—50 per cent.
A.M./6B	Do.	7.3	65.0	S. O.	I	+	+	N. H.	+	100	"
A.M./6C	Do.	7.3	65.0	S. O.	II	—	—	N. H.	—	..	Unclassified.
A.M./7A	Tank water (Shishmahal), drinking supply.	7.6	37.0	S. O.	I	+	+	N. H.	+	50 per cent	Tank 4B—25 per cent.

A.M./7B	Do.	7.6	37.0	S. T.	I	+	+	N. H.	+	50	”	Unclassified.
A.M./7C	Do.	7.6	37.0	S. O.	I	+	+	N. H.	+	50	”	Unclassified. (8314-20 per cent).
A.M./8A	Tank water (Hosainabad), bathing ghat.	8.0	2.0	S. O.	II	+	+	N. H.	+	Unclassified.
A.M./8B	Do.	8.0	2.0	S. T.	I (a)	+	+	N. H.	-	”
A.M./8C	Do.	8.0	2.0	S. T.	V (a)	+	+	N. H.	-	”
A.M./9A	Goomti river bathing ghat, drinking supply.	7.8	1.75	S. O.	II	+	+	N. H.	+	50 per cent	50 per cent	”
A.M./9B	Do.	7.8	1.75	S. T.	II	+	+	N. H.	+	”
A.M./9C	Do.	7.8	1.75	S. T.	II	+	+	N. H.	+	G. III-25 per cent.
A.M./9E	Do.	7.8	1.75	S. T.	II	+	+	N. H.	+	Unclassified (324/7-12.5 per cent).
A.M./10A	Stagnant pond, bathing only	8.2	7.5	S. O.	II	+	+	N. H.	+	Unclassified.
A.M./10B	Do.	8.2	7.5	S. O.	II	+	+	N. H.	+	”
A.M./11A	Goomti river temple ghat, drinking supply.	7.8	1.0	S. O.	I	+	+	P. H.	+	50 per cent	50 per cent	”
A.M./11B	Do.	7.8	1.0	S. T.	II	+	+	N. H.	+	50	”	”
A.M./13	Open pond (La Martiniere College)	8.0	2.0	S. T.	II	+	+	N. H.	+	50	”	”
A.M./14	Shallow well (Aminabad), drinking supply.	7.8	5.0	S. T.	II	+	+	N. H.	-	”

S. T. = Smooth translucent; S. O. = Smooth opaque; N. H. = Non-hæmolytic; P. H. = Partially hæmolytic. All strains were Gram negative, monoflagellate, actively motile vibrios and inagglutinable with 'O' group I serum. The 'Q' serological groups are those described by Taylor, Pandit and Read (*loc. cit.*). Strains agglutinating to less than 25 per cent of titre have not been taken as 'agglutinable' for the purpose of analysis of results.

TABLE VI.
United Provinces—Hardwar series.
Weekly samples from bathing ghat used by pilgrims.

Strain number.	Source.	Date of collection.	Colony characters.	BIOCHEMICAL REACTIONS.					SEROLOGICAL REACTIONS.	
				Hei- berg type.	Cholera red.	Indol.	Hæm- olysis.	V. P.	'H', + 'O', serum.	'O', serological group.
W./IA	River water (Hardwar), used for bathing and drinking.	1-6-1937	S. O.	IV	-	+	H.	-	..	Unclassified.
W./IE		1-6-1937	S. O.	VI (1)	-	-	N. H.	-	..	"
W./IIA		8-6-1937	S. O.	IV	-	+	P. H.	-	..	"
W./IID		8-6-1937	S. O.	IV	-	+	N. H.	-	..	"
W./IIE		8-6-1937	S. O.	II	+	+	N. H.	+	100 per cent	"
W./IIB		15-6-1937	S. O.	III	-	+	N. H.	-	..	"
W./IIC		15-6-1937	S. O.	IV	-	-	P. H.	+	..	"
W./IID		15-6-1937	S. O.	IV	-	+	N. H.	-	..	"
W./IVA		23-6-1937	S. O.	VI (1)	-	-	N. H.	-	..	"
W./IVB		23-6-1937	S. O.	V	-	+	N. H.	-	..	"
W./IVC		23-6-1937	S. T.	V (a)	+	+	N. H.	-	..	"
W./IVA		30-6-1937	S. T.	II	+	+	N. H.	+	..	"
W./IVB		30-6-1937	S. O.	VI (1)	-	-	N. H.	-	..	"

W./VC	Do.	..	30-6-1937	S. O.	I	+	+	N. H.	-	100 per cent	" (8314-20 per cent).
W./VI	Do.	..	7-7-1937	S. T.	II	+	+	N. H.	+	100 "	Unclassified.
W./VIIA	Do.	..	14-7-1937	S. O.	I	+	+	N. H.	+	50 "	8314-50 per cent.
W./VIIC	Do.	..	14-7-1937	S. T.	II	+	+	N. H.	+	50 "	Unclassified.
W./VIID	Do.	..	14-7-1937	S. O.	I	+	+	N. H.	+	..	"
W./VIHA	Do.	..	20-7-1937	S. O.	II	+	+	N. H.	-	50 per cent	"
W./VIHB	Do.	..	20-7-1937	S. O.	I	+	+	N. H.	-	50 "	"
W./IXA	Do.	..	27-7-1937	S. O.	II	+	+	N. H.	+	50 "	8314-25 per cent.
W./IXB	Do.	..	27-7-1937	S. O.	I	+	+	N. H.	+	100 "	Unclassified.
W./IXC	Do.	..	27-7-1937	S. O.	VI (1)	-	-	N. H.	-	..	"
W./XA	Do.	..	7-8-1937	S. T.	II	+	+	N. H.	+	..	1612-100 per cent.
W./XB	Do.	..	7-8-1937	S. O.	II	+	+	N. H.	+	100 per cent	Unclassified.
W./XE	Do.	..	7-8-1937	S. O.	I	+	+	N. H.	+	50 "	"
W./XIA	Do.	..	14-8-1937	S. O.	I	+	+	N. H.	-	50 "	Tank 4B-50 per cent.
W./XIB	Do.	..	14-8-1937	S. O.	I	+	+	N. H.	-	50 "	8314-25 per cent.
W./XIC	Do.	..	14-8-1937	S. O.	II	+	+	H.	-	50 "	Unclassified.
W./XID	Do.	..	14-8-1937	S. O.	I	+	+	N. H.	-	..	L/9/36-100 per cent.

S. T. = Smooth translucent; S. O. = Smooth opaque; H. = Hemolytic; N. H. = Non-hemolytic; P. H. = Partially hemolytic. All strains were Gram negative, monoflagellate, actively motile vibrios and inagglutinable with 'O' group I serum. The 'O' serological groups are those described by Taylor, Pandit and Read (*loc. cit.*). Strains agglutinating to less than 25 per cent of titre have not been taken as 'agglutinable' for the purpose of analysis of results.

TABLE VII.

United Provinces—Hardwar series.

Strains differing in biochemical or serological reactions.

Strain number.	BIOCHEMICAL REACTIONS.				SEROLOGICAL REACTIONS.	
	Heiberg type.	Indol.	Cholera red.	Haemolysis.	Inaba 'H' + 'O' — 50 per cent or over.	'O' serological group.
VIIA; XIB	I	+	+	N. H.	+	VIIA/8314—50 per cent.
VIIIB; IXB; XE	I	+	+	N. H.	+	XIB/8314—25 per cent.
XIA	I	+	+	N. H.	+	Unclassified.
XID	I	+	+	N. H.	+	Tank 4B—50 per cent.
XIC; IE; VI: VIIC; VIIA; XB.	II	+	+	N. H.	+	L./9/36—100 per cent.
VA	II	+	+	N. H.	—	Unclassified.
XA	II	+	+	N. H.	—	"
IXA	II	+	+	N. H.	+	1612—100 per cent.
IIIB	III	+	—	N. H.	—	8314—25 per cent.
IID; IIID	IV	+	—	N. H.	—	Unclassified.
IA	IV	+	—	H.	—	"
IIIC	IV	—	—	P. H.	—	"
IVB	V	+	—	N. H.	—	"
IVC	V	+	+	N. H.	—	"
IE; IVA; VB; IXC	VI	—	—	N. H.	—	"

H. = Haemolytic; N. H. = Non-haemolytic; P. H. = Partially haemolytic. All strains were Gram negative, monoflagellate, actively motile vibrios and inagglutinable with 'O' group I serum. The 'O' serological groups are those described by Taylor, Pandit and Read (*loc. cit.*). Strains agglutinating to less than 25 per cent of titre have not been taken as 'agglutinable' for the purpose of analysis of results.

TABLE VIII.

Punjab water samples—Cold-weather series.

Strain number.	Source.	pH.	Chlorine parts per 100,000.	Colony characters.	BIOCHEMICAL REACTIONS.				SEROLOGICAL REACTIONS.	
					Hei-berg type.	Cholera red.	Indol.	Hæm-olysis.	Inaba 'H' + 'O' serum.	'O' serological group.
W.1/1	Kalka spring water, used for drinking	7.8	1.0	S. O.	I	—	+	H.	..	Unclassified.
W.2/1	Stagnant pond, not used for drinking	8.0	2.0	S. O.	I	—	—	H.	..	"
W.3/1	Pinjour tank water, used for drinking	7.2	1.5	S. O.	V	—	+	N. H.	..	"
W.4/1	Pinjour tank water, not used for drinking.	7.5	1.5	S. O.	IV	—	+	N. H.	..	"
W.4/2	Do.	7.5	1.5	S. T.	V	—	+	N. H.	..	"
W.4/20	Do.	7.5	1.5	S. T.	VI	—	—	N. H.	..	"
W.5/2	Shallow well (Chandigarh), used for drinking.	7.4	1.0	S. O.	V	—	+	N. H.
W.5/21	Do.	7.4	1.0	S. O.	I	—	+	H.

S. T. = Smooth translucent; S. O. = Smooth opaque; R. O. = Rough opaque; H. = Hæmolytic; N. H. = Non-hæmolytic. All strains were Gram negative, monoflagellate, actively motile vibrios and inagglutinable with 'O' group I serum. The 'O' serological groups are those described by Taylor, Pandit and Read (*loc. cit.*). Strains agglutinating to less than 25 per cent of titre have not been taken as 'agglutinable' for the purpose of analysis of results.

TABLE VIII—*concl'd.*

Strain number.	Source.	pH.	Chlorine parts per 100,000.	Colony characters.	BIOCHEMICAL REACTIONS.				SEROLOGICAL REACTIONS.	
					Hei-berg type.	Cholera red.	Indol.	Hæmoly- sis.	'H' + 'O' serum.	'O' serological group.
W.5/22	Shallow well (Chandigarh), used for drinking.	7.4	1.0	S. O.	IV	—	+	N. H.	..	G. III—25 per cent.
W.6/2	Shallow well (Panchkoola), used for drinking.	7.6	2.0	S. T.	VI	—	—	N. H.	..	Unclassified.
W.6/23	Do.	7.6	2.0	S. O.	I	—	+	H.	..	"
W.6/24	Do.	7.6	2.0	S. O.	V	—	+	N. H.	..	8314—100 per cent.
W.7/6	Shallow well (Mubarakpur), used for drinking.	7.6	2.0	S. O.	VI	—	—	N. H.	..	Unclassified.
W.7/30	Do.	7.6	2.0	S. T.	V	—	+	N. H.	..	8314—100 per cent.
W.8/2	Stagnant pond (Mubarakpur), not used for drinking.	7.4	4.0	S. T.	I	—	—	H.	..	"
W.9/1	Shallow well (Basi), used for drinking	7.8	9.0	S. O.	IV	—	+	N. H.	..	"
W.9/2	Do.	7.8	9.0	S. O.	I	—	+	H.	..	"
W.9/4	Do.	7.8	9.0	S. T.	VI	—	—	N. H.	..	1805/1—25 per cent.
W.10/3	Tank water (Lalru), not used for drinking.	7.2	2.5	S. O.	IV	—	—	H.	..	Unclassified.

W.11/1	Shallow well (Sakini), used for drinking.	7.6	17.5	S. O.	I	—	—	N. H.	..	"
W.11/2	Do.	7.6	17.5	R. O.	VI	—	—	N. H.	..	"
W.12/2	Shallow well (Shahabad), used for drinking.	7.5	6.25	S. O.	VI	—	—	N. H.	..	1905/1—100 per cent.
W.14/4	Shallow well (Jhanjhari), drinking supply for Chamars.	7.6	12.0	S. O.	VI	—	—	N. H.	..	Unclassified.
W.14/25	Do.	7.6	12.0	S. O.	V	—	—	N. H.	..	"
W.15/26	Shallow well (Jhanjhari), drinking supply for Hindus.	7.4	40.0	S. T.	III	—	+	N. H.	..	"
W.15/27	Do.	7.4	40.0	S. O.	I	—	—	H.	..	2943—50 per cent.
W.15/28	Do.	7.4	40.0	S. O.	V	—	—	N. H.	..	Unclassified.
W.16/2	Shallow well (Panipat), not used for drinking.	7.6	75.0	S. T.	II	—	—	H.	..	"
W.16/7	Do.	7.6	75.0	R. O.	VI	—	—	N. H.	..	"
W.17/1	Shallow well (Karnal City), drinking supply for sweepers.	7.6	4.0	S. T.	II	—	—	H.	..	"
W.17/5	Do.	7.6	4.0	S. T.	VI	—	—	N. H.	..	"
W.17/29	Do.	7.6	4.0	S. O.	I	—	—	H.	..	"

S. T. = Smooth translucent; S. O. = Smooth opaque; R. O. = Rough opaque; H. = Hemolytic; N. H. = Non-hemolytic. All strains were Gram negative, monoflagellate, actively motile vibrios and inagglutinable with 'O' group I serum. The 'O' serological groups are those described by Taylor, Pandit and Read (*loc. cit.*). Strains agglutinating to less than 25 per cent of titre have not been taken as 'agglutinable' for the purpose of analysis of results.

TABLE IX.

Summary of results of examination of natural water sources in Northern India for vibrios during the hot-weather and monsoon periods, 1937.

Date of collection.	Area of collection.	Number of sources examined.	Number of sources positive.	Number of strains examined.	SUGAR REACTIONS (HEIBERG TYPES).						Number of strains aggl. with Inaba, 'H' + 'O' serum (50 per cent or over).	Number of sources yielding H.-Ag. vibrios.	NUMBER OF NAG STRAINS HAVING SEROLOGICAL RELATIONSHIP WITH			Number of sources yielding vibrios having serological relationship with NAG case strains.
					I	II	III	IV	V	VI			NAG vibrios from cases.	NAG vibrios from carriers.	NAG vibrios from water.	
April 1937	Ambala and Karnal districts.	19	19	35	8	18	0	7	0	2	12	8	5	2	0	5
May 1937	Lucknow ..	13	13	26	11	14	0	0	1	0	14	9	1	2	1	1
July 1937	Ambala and Karnal districts.	21	21	38	8	14	3	3	0	10	12	9	9	4	0	7
August 1937	Karnal district.	2	2	7	4	3	0	0	0	0	2	2	1	2	1	1
August 1937	Multan and Ambala districts.	19	19	24	8	10	2	0	0	4	7	5	4	1	0	3
TOTAL ..		74	74	130	39	59	5	10	1	16	47	33	20	11	2	17
PERCENTAGE		..	100	..	30	45.38	3.8	7.7	0.77	12.3	36.1	25.38	15.38	8.46	1.54	13.0

TABLE XI.
United Provinces—Hardwar series.

Date of collection.	Area of collection.	Number of samples examined.			SUGAR REACTIONS (HEIBERG TYPES).						Number of strains aggl. with Inaba, H ⁺ , O ⁺ serum (50 per cent or over).		Number of samples yielding H.-Ag. vibrios.		NUMBER OF NAG STRAINS HAVING SEROLOGICAL RELATIONSHIP WITH			Number of samples yielding vibrios serologically related to case and carrier strains.
		Number of samples examined.	Number of samples positive.	Number of strains examined.	I	II	III	IV	V	VI	NAG vibrios from cases.	NAG vibrios from carriers.	NAG vibrios from water.					
June, July and August 1937.	Hardwar ..	11	11	30	9	9	1	5	2	4	14	2	3	1	2	
PERCENTAGE		..	100	..	30	30	3.3	16.7	6.6	13.3	46.6	6.6	10	3.3	18.2	

TABLE XII.

Water vibrio strains showing 'O' serological relationship with case strains of types other than 'O' group I.

Strain number.	Agglutinating with Inaba 'H' + 'O' serum (50 per cent to 100 per cent).	Agglutinating with 'O' group serum number.*		Origin of type strain.
12/2	—	1805/1	100 per cent	Calcutta case.
2A	+	1805/1	50 "	"
10D	+	1805/1	50 "	"
9/4	—	1805/1	25 "	"
17A	+	324/7	50 "	"
11B	+	324/7	25 "	"
L1	+	324/7	25 "	"
XA	—	1612	100 "	"
D1	+	1612	25 "	"
2B	—	630/3T	50 "	"
14A	+	GVI	100 "	"
L2A	—	627/29/2	25 "	"
4D	—	L/1	100 "	Lahore case.
8A	+	L/9	100 "	"
18A	—	L/9	100 "	"
XID	+	L/9	100 "	"
12B	—	984	25 "	Assam case.
4B	—	3067	100 "	"
5/22	—	GIII	25 "	Nanking case.

* The 'O' group sera used are those described by Taylor, Pandit and Read (*loc. cit.*).

TABLE XIII.

Water vibrio strains showing 'O' serological relationship with carrier strains of types other than 'O' group I.

Strain number.	Agglutination with Inaba 'H' + 'O' serum (50 per cent to 100 per cent).	Agglutination with 'O' group serum number.*			Origin of type strain.
2C	—	8314	100	per cent	Calcutta carrier.
6/24	—	8314	100	"	"
7/30	—	8314	100	"	"
VIIA	+	8314	50	"	"
14A	—	8314	50	"	"
13B	+	8314	25	"	"
15B	+	8314	25	"	"
18B	+	8314	25	"	"
XIB	+	8314	25	"	"
IXA	+	8314	25	"	"
L10	—	5780	100	"	"
12D	—	5780	50	"	"
6A	+	2943	50	"	"
15/27	—	2943	50	"	"
3B	+	2898	50	"	"
1A	+	2898	25	"	"

* The 'O' group sera used are those described by Taylor, Pandit and Read (*loc. cit.*).

THE DISTRIBUTION AND CHARACTERISTICS OF VIBRIOS ISOLATED FROM CERTAIN NON-HUMAN SOURCES IN CALCUTTA.

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THE *Vibrio cholerae*, the universally accepted causative organism of cholera, has been the subject of very careful study by a number of bacteriologists but the large number of species of vibrios which have been isolated both from human and other sources and which resemble closely the *V. cholerae* in their general biological characters have not been systematically studied. Some of these vibrios exhibit slight differences in size or in certain biochemical properties and have been given names which refer to certain biological peculiarities or more often to the locality where the vibrios were found. The majority of such vibrios cannot be distinguished from *V. cholerae* except in their serological reactions. A certain percentage of such vibrios agglutinate with a cholera serum containing both the 'H' and 'O' agglutinins but they do not agglutinate with pure 'O' serum of the type group I of Gardner and Venkatraman (1935). Although the 'O' inagglutinable vibrios are found in sewage or in polluted waters, similar vibrios are occasionally found in the stools of cholera patients, carriers, or contacts and their presence sometimes causes confusion in making a bacteriological diagnosis. Because of the very close resemblance of these vibrios to *V. cholerae* and the common 'H' antigen possessed

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by some of the 'O' inagglutinable vibrios, certain workers have attributed choleraenic properties to such 'O' inagglutinable vibrios but this is based on data which are as yet inconclusive. As a preliminary to a detailed systematic study of the vibrios which are found in nature a survey was made of the distribution and some of the characteristics of vibrios occurring in certain non-human sources.

The following sources were examined :—

(1) Three hundred samples of surface waters were collected periodically from the beginning of July 1937 to the end of February 1938. There were 220 samples collected from certain surface tanks situated in different parts of Calcutta and the outlying areas and 80 samples of water collected from different parts of the River Hooghly as it passes through Calcutta. (2) Forty-six samples of different tubed water-supply. (3) Six hundred and forty flies caught individually in separate sterile tubes, and 94 catches of cockroaches.

The methods employed for the isolation of vibrios from waters and other sources are summarized below :—

(1) *Technique for the isolation of vibrios in waters.*—Approximately 180 c.c. of the sample of water were collected in flasks containing 20 c.c. of 10 per cent peptone water containing 5 per cent sodium chloride. The flasks were left at room temperature in the dark overnight and the next morning 1 c.c. of the fluid from the flasks was transferred to a tube of peptone water. After six hours' incubation at 37°C. the surface growth from the tubes was inoculated on freshly prepared Aronson's medium. Three vibrio colonies were picked up from each positive plate and were studied for their morphology, biochemical and serological reactions. At first the samples of water which gave no vibrio colonies were examined after further peptone-water enhancement. It was found, however, that the samples which were negative after the first enhancement generally failed to show any vibrios after further enhancement and this method of examination was discontinued. The first hundred samples were inoculated both on Aronson's medium and on $\frac{1}{2}$ per cent bile-salt agar. Ninety-eight per cent of the samples inoculated on Aronson's medium showed the presence of vibrios, whereas only 55 per cent of the samples of the same series inoculated on bile-salt agar gave vibrio colonies. There was often growth of spreading colonies on the bile-salt agar plates and this made the isolation of vibrios difficult, whereas on Aronson's medium the number of vibrio colonies was proportionately greater and there was entire freedom from 'spreaders' on the plate. As the isolation of vibrios was distinctly better on Aronson's medium than on bile-salt agar, the latter medium was not used for the rest of the series.

(2) *Technique for the isolation of vibrios in flies.*—The flies were caught in separate test-tubes and were put in tubes containing 10 c.c. of 1 per cent peptone water. The tubes were left overnight at room temperature and the next morning 1 c.c. of the surface growth was inoculated into fresh peptone water and incubated for six hours at 37°C. The surface growth from the second tube was plated on Aronson's medium. Similar methods were employed for the isolation of vibrios in cockroaches.

The results of the examinations are given below :—

(A) *Vibrios in waters.*

TABLE I.

Showing the source of the samples, the total number of samples examined from each source, and the number and percentage in which vibrios were isolated.

Source.	Number of samples examined.	Number and percentage of samples from which vibrios were isolated.
Open surface tanks ..	220	189 (86 per cent).
River Hooghly ..	80	80 (100 „ „).

It will be seen that vibrios were found in every sample of water collected from the River Hooghly. Quantitative examinations were made to determine the minimum amount of water which would give vibrio colonies. Vibrios could always be isolated from 50 c.c. and occasionally from 5 c.c. of the samples. A sufficient number of quantitative examinations has not been made to determine any seasonal variation in the vibrio content of the waters.

The tank waters collected in July, August, September, and October 1937, and January and February 1938 showed the presence of vibrios in 96 per cent of the samples examined. The tank waters collected during November and December 1937 gave vibrios in 43 and 45 per cent of the samples examined.

Three hundred and eighty colonies of vibrios isolated from different samples of water were studied. The appearance of the colonies varied from the typical readily recognizable type to the rough rugose type of colony. Biochemically they were of the following Heiberg's types :—

Type ..	I	II	III	IV	V	VI
	160	201	9	2	3	5

The majority (about 75 per cent) of the samples gave vibrios of more than one Heiberg's type.

All strains were tested with standard Inaba 'O' and Inaba 'H' and 'O' sera. No strain was agglutinable with Inaba 'O' serum and 20 or 5.2 per cent of the 380 strains were agglutinable with Inaba 'H' and 'O' serum to more than 50 per cent of the titre of the serum. Twelve strains of vibrios isolated from different samples of waters and which were agglutinable with 'H' and 'O' cholera serum were further studied. It was found that in the majority of the strains the 'H' relationship to *V. cholerae* was only partial and that there was a residual 'H' antigen which was specific to the 'O' inagglutinable vibrio tested.

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Three of the 17 samples of laboratory tap-water showed the presence of 'O' inagglutinable vibrios, whereas vibrios were absent from 12 samples of water collected direct from the municipal water hydrants. This suggested that the vibrios found in the laboratory tap-water were the result of contamination occurring during the storage of water. Samples of dust from the street, the floors of the laboratory, and from the top of cupboards showed the presence of vibrios in the majority of the samples. Four samples of tube-well water collected direct from the supply pump gave no vibrio colonies, whereas a sample of tube-well water stored in an overhead tank contained vibrios.

Eight of 13 samples of waters collected from different municipal markets and used for washing vegetables and fish showed the presence of vibrios. Vibrios were found in the intestines of 12 fishes examined.

(B) *Vibrios in flies and cockroaches.*

Two collections of 320 flies each were examined for the presence of vibrios on their bodies. The first collection was made during the latter part of the rainy season and the second collection made during the dry and cold weather. The results of these examinations are given in Table II :—

TABLE II.

Number of flies examined.	Flies collected in	Number and percentage of flies from which vibrios were isolated.	Number of flies from which 'H' agglutinable vibrios were isolated.
320	August 1937	62 (19.2 per cent)	10
320	December 1937	22 (6.8 „ „)	Nil.

No 'O' agglutinable vibrios were isolated.

The vibrios isolated from the two collections of flies belonged to the following Heiberg's types :—

Type	..	I	II	III	IV	V	VI
August catch	..	53	4	5	0	0	0
December catch	..	14	3	2	3	0	0

A batch of 94 cockroaches caught during August 1937 showed the presence of vibrios in 16 or 17 per cent. These vibrios were inagglutinable with Inaba 'O' and Inaba 'H' and 'O' sera and biochemically belonged to Heiberg's type I (87 per cent) and type II (13 per cent).,

SUMMARY.

1. Three hundred samples of surface waters collected from the River Hooghly and from certain surface tanks in Calcutta were examined for the

presence of vibrios. Vibrios were isolated from all the 80 samples of the river waters and from 86 per cent of the 220 tank waters examined.

2. Vibrios were absent in tube-well water and in the municipal tubed supply of drinking water. Vibrios were found in these waters after storage in domestic tanks suggesting the contamination of the water during storage.

3. Vibrios were found in 62 or 19 per cent of 320 flies caught in different parts of Calcutta during the month of August 1937 and in 22 or 6·8 per cent of 320 flies caught in December 1937. Vibrios were found in 16 or 17 per cent of 94 cockroaches examined.

4. The vibrios isolated from different samples of water, from flies and cockroaches were all inagglutinable with Inaba 'O' serum. Seven per cent of the vibrios isolated from various sources showed 'H' relationship with *V. cholerae*. A detailed serological study of 12 of the 'H' agglutinable strains showed that in the majority there is more than one 'H' antigen, one of which is common to the 'H' antigen of *V. cholerae*.

5. Biochemically the majority of the strains of vibrios isolated belonged to Heiberg's types I and II and very few to the other types.

6. The vibrios isolated from the non-human sources examined resemble in their morphology and in their main biochemical reactions the *V. cholerae*, and can be distinguished only by their inagglutinability with Inaba 'O' serum. The method of isolation must be stressed for there is evidence to suggest that with different methods of isolation other species of vibrios can be obtained.

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VIBRIOS IN NATURAL WATER SOURCES IN ASSAM.

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THE method employed for isolating vibrios from water differed from routine methods essentially in cultivation at or near a pH of 9·0. An eight-ounce stoppered bottle containing 20 c.c. of 10-fold strength peptone water at a pH of 9·0, previously sterilized, was filled with the water under examination to the 200-c.c. mark—file mark on the side of the bottle—and sent to the laboratory for examination. No attempt was made to readjust the pH to 9·0; but in several instances examined, the pH of the contents was found to be not less than 8·0 and usually in the neighbourhood of 9·0. The bottles were incubated for 18 hours, at the end of which a large loopful of the contents from the surface was transferred to a tube of peptone water. After 4 hours and sometimes after 18 hours' incubation, a small loopful from the surface of this was plated on agar and Aronson plates and suspicious colonies picked up.

One hundred and five samples from wells, tanks, streams, ponds, and other collections of water were examined. Of these 95 or 90·5 per cent were positive for vibrios. The strains, classified according to the Heiberg types, using the six sugars, glucose, lactose, mannite, saccharose, mannose, and arabinose, fell into the following groups :—

I	II	III	IV	V	VI(1)	VI(2)	Gas former	Total
9	11	9	4	7	88	10	1	139

The type VI(1) vibrios did not ferment any of the sugars and type VI(2) usually fermented glucose and mannite but not any of the other sugars. Certain strains, however, that fermented glucose and lactose only have been classed here as

type VI(2). The gas-forming vibrio was monotrichate and gave the biochemical reactions of type I. In 64 samples there was only one biochemical type of vibrio, in 26 two types, and in 5 more than two types, one of which contained as many as 5 biochemical types.

The VI(1) type of vibrio alone was found in 60 samples and in combination with other types in 28 others. Fifty of these strains were examined in greater detail as regards staining for flagella and other biochemical reactions. The V-P (original and the α -naphthol modification) and the cholera-red reactions and the indol test were all negative. On the basis of gelatin liquefaction, nitrate reduction, and reaction of litmus milk, the 50 strains were distributed as follows:—

GELATIN LIQUEFACTION.		NITRATE REDUCTION.		LITMUS MILK.	
+	—	+	—	Alkaline	Not alkaline.
9	41	41	9	46	4*

* All the 4 strains gave an initial alkalinity followed by acidity in the case of two, and decolorization and peptonization in the remaining two.

Thirty-three strains were monotrichate and 17 lophotrichate. *Bact. faecalis alkaligenes*, which gives the same fermentation reactions, did not reduce nitrates or liquefy gelatin and produced alkalinity in milk. Identical reactions were given by 5 out of the 50 VI(1) type vibrio strains and these were only differentiated from *Bact. faecalis alkaligenes* by flagellar staining, two being found to be monotrichate and three lophotrichate. It may be mentioned here that most of the strains were bacillary and only a few had the vibrio shape.

The samples of water were obtained from both the valleys of Assam—the Brahmaputra and Surma Valleys—as well as the hill district of Khasi and Jaintia Hills. Fifty-four of the samples were collected during the hot months May to July and the remaining samples during the cold months December to February. There was no significant difference either in the percentage isolation or the types of vibrios for those periods.

SUMMARY.

1. Vibrios were isolated from 90 per cent of open-water sources in Assam, including wells, tanks, streams, and ponds.

2. Strains were obtained which belonged to all six Heiberg fermentation types. The majority belonged to the non-fermenting type VI. Certain of the characters of type VI vibrios have been studied.

3. No material difference was noted in the incidence of vibrios or in their characters during the cold-weather as compared with the hot-weather period.

STUDIES ON THE SPECIFIC POLYSACCHARIDES OF THE VIBRIOS.

Part II.

CHEMISTRY AND SEROLOGY.

BY

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FROM an extended study of the chemical composition of the vibrios, Linton, Shrivastava and Mitra (1935) were able to classify them into six groups, which corresponded with their origin, biochemical activities, and metabolism. Our later work has included an attempt to determine if a serological basis for this chemical classification could be found, and it is the purpose of this paper to report on these experiments. It was also hoped that certain discrepancies would be cleared up with the use of the precipitin test, such as, for example, the position of the El Tor strains, which can easily be distinguished from vibrios from cases of cholera by chemical means, but not by means of 'O' agglutination (Gardner and Venkatraman, 1935). Furthermore, vibrios belonging to chemical group II from cases of cholera were found to contain the same polysaccharide as those of chemical group III, from water, but the two were quite distinct in the agglutination test.

In the present communication we are reporting a chemical and serological study of the vibrio polysaccharides. These have been isolated by a technique

developed by Shrivastava and Seal (1937) from the centrifugates of peptone-water cultures. With this method all drastic treatment of the polysaccharides is avoided. In the paper just referred to, it was shown that by the use of such polysaccharides in the precipitin test, one could readily distinguish El Tor strains from vibrios isolated from cholera cases, although they were identical when tested with 'O' agglutinating sera; also group I strains could be distinguished from group VI. We are now reporting a continuation of this work, using strains from all the chemical groups.

DETAILS OF THE STRAINS USED.

In all, about two dozen strains have been worked with. Strains 176, 179, 196, 198, 200, and 202 were obtained from cases of cholera in the earlier part of the 1937 epidemic in Calcutta, and strains 315, 319, 322, 330, and 334 from cases during the latter part of the same epidemic. All these strains were agglutinated by Inaba 'O' serum to significant titres. H4, H5, and H25 were isolated from hospital patients not suffering from cholera. W115/5, W132, W138/2, W139/2, W164, W182, and W171 were isolated from water sources in and around Calcutta and were non-agglutinable with Inaba 'O' antiserum. All these strains were used in our work within six weeks of their isolation and accordingly had not been maintained under artificial laboratory conditions for a long time. No. 1894 is a vibrio isolated from a tank in Diamond Harbour in 1935 and is non-agglutinable. We obtained all the above strains through the courtesy of Major C. L. Pasricha, I.M.S. Strain Inaba 'T' is the classical Japanese case strain, kindly sent to us by Colonel J. Taylor, I.M.S.

The specific polysaccharide was isolated and the chemical group determined in each particular case at about the same time. The chemical group was determined by identifying the type of protein and polysaccharide present in the strain. The carbohydrate was identified by the modified process of Linton, Mitra and Seal (1936) and the protein by the process developed by Linton, Mitra and Shrivastava (1934), with a slight modification in the mode of preparing the solutions for polarimetric readings. After dissolving the protein in NaOH solution of required strength, the solution was incubated at 37°C. for 24 hours and then passed through a Chamberland L-2 candle. The readings of the optical rotation of the clear solution were taken after this at specified intervals. Nitrogen was estimated by means of the Kjeldahl method and the percentage content of protein in the solution calculated from it.

PREPARATION AND CHEMICAL PROPERTIES OF THE POLYSACCHARIDES.

The method of preparing the specific polysaccharides was essentially the same as already reported (Shrivastava and Seal, *loc. cit.*; Linton, Shrivastava and Seal, 1938). It was found, however, that better preparations were obtained if the polysaccharide solution was shaken with CHCl_3 , etc., once or twice, about half an hour each time, in the final stages. In this way water-clear solutions were obtained for the final isolation. For any particular polysaccharide from 6 to 8 shakings of half an hour duration each were made.

All the polysaccharides were biuret negative and gave positive Molisch reactions in very high dilutions. They were free from phosphates. Some of the polysaccharides of each of the three types were found to contain organically bound phosphorus and traces of sulphur as well.

Table I gives the results obtained with case strains recently isolated, during the beginning as well as toward the end of the epidemic of cholera in 1937; together with a case strain, Inaba 'T', which has been maintained under artificial conditions for a long time. All these strains belong either to group I or group II of the chemical classification. The specific rotations in the majority of cases lie between $+80^{\circ}$ and $+95^{\circ}$. No. 179 has a rather low and No. 196 a comparatively high figure. The nitrogen values in the cases of strains isolated in the initial stages of the epidemic are high (4.7 to 9.1 per cent). Compared to these, the strains from the latter part of the epidemic have lower figures for nitrogen, which range from 1.4 to 3.2 per cent. The values for the acetyl group in the first set of strains are lower (0.0 to 2.6 per cent) than in the case of the second set (2.6 to 4.7 per cent). The two sets of strains are also different in their figures for maximum hydrolysis in N/1 H_2SO_4 , that for the earlier strains ranging from 31.2 to 46.1 per cent as against 54.8 to 63.4 per cent in the later strains. It was interesting to note that polysaccharides of strains from the first part of the epidemic developed a heavy opalescence upon the addition of H_2SO_4 to their aqueous solutions, while polysaccharides of strains from the latter part of the epidemic gave little or no opalescence. Furthermore, it was found that if the polysaccharides which showed the strong opalescence were dissolved in N/10 acetic acid and refluxed in boiling water, a flocculent precipitate appeared within 30 minutes. This precipitate was ether-soluble, and biuret and Molisch negative. Since the polysaccharides had a high nitrogen content and contained phosphorus, it is highly probable that they are a carbohydrate phospholipoid complex. It was also noted that polysaccharides having this lipoid complex did not hydrolyse to a high percentage in N/1 H_2SO_4 , and have a comparatively large amount of nitrogen.

Strain 334 which had been isolated in the latter part of the epidemic from a severe case of cholera has behaved differently from other strains isolated during the same period. It has a high nitrogen content of 6.9 per cent and a low figure for maximum hydrolysis. It also developed a heavy opalescence on the addition of acid to its aqueous solution. In these characteristics it resembles polysaccharides from strains isolated in the earlier part of the epidemic.

It is interesting to compare the figures for Inaba 'T' with the corresponding ones for the strains discussed above. In every respect, except the acetyl-group figure, which is much higher (6.3 per cent), it is in line with the second set of strains isolated in the latter part of the epidemic.

Results obtained with water and carrier strains are given in Table II. Of the 8 water strains, W132, W138/2, W139/2, W164, and W182 belong to chemical group III, and W171 is a strain of mixed groups III and IV. W115/5 belongs to group IV and No. 1894 is again a mixed strain falling into groups IV and V, with group V predominating. Of the 3 carrier strains examined, H5 falls in group IV and H4 and H25 fall into groups IV and V.

There is a much wider range of variation in the figures for specific rotations of the polysaccharides from strains belonging to groups III, IV, and V than in the case of groups I and II. The lowest figure ($+37.9^{\circ}$) is found in polysaccharide W164 and the highest ($+118.4^{\circ}$) in No. 1894. The range of figures for nitrogen is from 1.5 to 3.2 per cent which is of the same order as in the strains isolated in the latter part of the epidemic. The values of maximum hydrolysis are usually high, ranging from 68.6 per cent for H25 to 41.5 per cent for H4. All the strains of groups III, IV, and V examined have uniformly been found not to develop opalescence upon the addition of acid to their polysaccharide solutions. Accordingly, they do not contain the lipid component which we have found to be present in freshly isolated strains from cases of cholera in the beginning of an epidemic.

Table II also gives the figures for Inaba variant polysaccharide, for comparison. They have already been reported (Linton, Shrivastava and Seal, *loc. cit.*). Nitrogen and hydrolysis figures are like those of the strains of groups III to V. The acetyl-group content is high at 12.8 per cent. The strain does not possess the lipid factor.

DISCUSSION OF THE CHEMICAL FINDINGS.

From a study of the data presented in Tables I and II, it is clear that the vibrio strains isolated in the beginning of an epidemic and examined soon after their isolation are chemically distinct from those isolated from cases in the latter part of the epidemic, or from water and other sources. The latter strains seem to have a higher acetyl-group content, lower nitrogen, and a higher percentage of hydrolysis than the former ones. It would appear that in the case of the vibrios, a high acetyl content is not a sign of pathogenicity, since water and carrier strains have much higher acetylation than strains from cases.

Of more significance, however, is the observation of the presence of a lipid constituent in strains isolated in the early part of an epidemic. This factor is either much diminished or completely absent in strains from the latter part of the epidemic and is totally wanting in non-cholera strains. The lipid complex is also absent in strain Inaba 'T' which has been cultivated on artificial media for a long time, although originally from a case of cholera. There appears to be some relationship between this complex and pathogenicity, and the observation of its presence recalls that of Topley *et al.* (1937), on mouse-typhoid and other strains, where a phospho-lipoid compound has been found in antigenic fractions. These lipid fractions in the vibrios need further investigation.

The results obtained in this part of the work will be discussed further below in the light of the serological findings.

SEROLOGY OF THE SPECIFIC POLYSACCHARIDES.

Preparation of antisera.—Eighteen-hour cultures of vibrios on agar slopes were washed in normal saline and after adjusting the concentration of organisms per c.c., were injected intravenously into rabbits. The first dose was 500 millions of living vibrios, but it was gradually increased to 5,000 millions during the course of the following injections. One course consisted of 3 injections on alternate days, followed by a rest for a week. Usually three such courses had to be given, but in

certain cases 5 or 6 courses were required before good precipitin titres were obtained. Animals were bled a week after the last injection, and the sera diluted with one volume of sterile normal saline and preserved in a refrigerator after adding Merthiolate to make its final concentration 1 : 15,000.

Precipitin reactions.—Tubes slightly longer and less tapering than the usual Dreyer's tubes were used for carrying out the tests. Solutions of polysaccharides of the required dilutions and the antisera were mixed in equal volumes in the tubes and incubated in the water-bath at 52°C. After 2 hours they were removed and put into the refrigerator overnight and readings taken the following morning. The final serum dilution in every case was 1 in 10.

Each antiserum was set up against solutions of various dilutions of the homologous as well as of all the heterologous polysaccharides. The results with the homologous polysaccharides are given in Table III. The dilutions of polysaccharides usually tried were from 1 : 2,000 to 1 : 4 millions, each higher dilution being twice the preceding. In special cases dilutions higher than 4 millions were also tried.

In general, the homologous precipitin titres with these antisera ranged from 1 : 250,000 to 1 : 2 millions. Three of the strains fell below this range, two of them probably because too few injections were given. The third, No. 330, did not show a good titre even after five courses of injections, and these poor titres were obtained in three successive rabbits, although numerous injections were given to each. It is possible that some factor in the strain itself, rather than in the animals, is responsible for this result. Three of the strains showed titres of 4 millions, 4 millions, and 16 millions, respectively.

The results with heterologous reactions are given in Tables IV and VI. The heterologous titres are expressed as percentages of the homologous titre of the serum, i.e., the homologous titre is taken as 100 per cent and the heterologous titres based as percentages on this figure. Any titres below 12.5 per cent have been arbitrarily put down as negative. It may be pointed out in explanation of this course that even in those cases where the homologous titres are four millions or above, the reactions put down as negative did not in any case exceed 5 or 6 per cent.

Table IV gives the percentage titres of all the antisera against the polysaccharides from strains of groups I and II. It will be seen that these polysaccharides react neither with the antisera of water strains, nor with the antisera for H5, 1894, and Inaba variant, which belong to groups IV, IV and V mixed, and VI, respectively. There is a certain amount of significant cross-reaction of H4 and H25 antisera with groups I and II polysaccharide, for which we cannot find any explanation at this stage.

While discussing the chemical data above it was pointed out that strains isolated in the first part of an epidemic differed from those isolated in the latter part with the exception of strain 334, which had been isolated in the latter part. The serological data in Table IV show again the anomalous position of this strain, and the distinctness of the whole 'early' group of organisms. Designating these early strains and strain 334 as group A and the later ones as group B, it will be seen that there are 6 antisera in group A, which have been set up against 7

polysaccharides of the same group, giving a total of 42 reactions. The antisera of group A have also been set up against 5 polysaccharides of group B, and the number of heterologous reactions is thus 30. Similarly, the homologous cross-reactions in group B number 25 and the heterologous number 35. If we tabulate the various degrees of reaction and calculate the percentages of the total number of cross-reactions in any particular set, we get the result shown in Table V.

It is evident from Table V that the figures for the homologous reactions in both A and B are similar in that the highest figures in both cases are for the 100 per cent reaction. For the heterologous reactions, however, the case is reverse. The figures for the 100 per cent reactions are the lowest, whereas the figures for no reaction are the highest. There is accordingly a distinct degree of group specificity in the groups A and B and although cross-reactions do occur the two groups are quite distinct. The chemical basis for the cross-reactions are discussed below.

Table VI gives the reactions of all the antisera against the polysaccharides belonging to groups III, IV, V, and VI.

The results given in Table VI show that the water strains of group III are distinct from the other strains. Similarly, the polysaccharides from groups IV, IV and V mixed, and VI do not react with the antisera to groups I and II. The only marked exceptions are strains H4 and H25. There is almost no cross-reaction within group III, but there is a fair amount within the groups IV and IV and V mixed. The polysaccharide from Inaba variant (group VI) reacts only with its own antiserum.

ATTEMPTS TO PREPARE ANTISERA TO POLYSACCHARIDES.

Various attempts were made to prepare antisera to polysaccharides to determine if such sera would be specific. The chosen polysaccharide was well mixed with its homologous antiserum and after standing in the cold for 24 hours was injected intravenously into rabbits. The dose began with 1 c.c. made equivalent to 1 mg. of polysaccharide, and injections were given on alternate days. The dose was gradually increased to 8 mg. during the course of the next 6 or 8 weeks. Rest for a week was allowed after every three injections. As no titres were obtained by this method, the polysaccharide was mixed with ox-serum and injected into rabbits as before but this method also did not yield sera having any definite precipitin antibodies. Better results might be achieved by using other experimental animals, e.g., mice, as in the case of pneumococcus polysaccharide (Avery and Goebel, 1933), and some other route of injection. In this connection it is of interest to note that some of the polysaccharides were highly toxic to rabbits, and these were frequently, although not exclusively, of the lipid-containing type.

DISCUSSION.

The chemical and serological results described above have confirmed our previous findings that cholera strains are quite distinct from strains from other sources. It has been established that the two groups from cholera cases cross-react to a certain extent, but not completely, thus confirming their relationship as

shown in the chemical studies. These two groups are chemically and serologically distinct from groups III, IV, and VI. Group III is distinct from all the other groups. Two strains of groups IV and V react with groups I and II, while the third (1894) does not react.

Among the cholera strains, the presence of two types has been demonstrated. They differ from each other both chemically and serologically. One factor responsible for these differences is a lipid-polysaccharide complex, which appears to be present only in strains freshly isolated in the early part of an epidemic. If we represent the polysaccharides of lipid-containing strains belonging to groups I and II by $C_1 + L$ and $C_2 + L$, where C_1 and C_2 stand for the two types of carbohydrates, and L stands for the lipid component, we see that $C_2 + L$ reacts better with antisera belonging to strains containing $C_1 + L$ than with antisera against strains containing C_2 only. Thus, it would appear that in the lipid-polysaccharide complex, it is the lipid portion that dominates specificity. When the lipid is absent the specificity is determined by the polysaccharide. More detailed work is necessary on the lipid factor to determine both its relationship to virulence and to specificity.

It is of interest to observe that the strain Inaba 'T', which is an old laboratory strain originating from a case of cholera, is not the same in all respects as a freshly isolated strain. The lipid complex is absent, and the nitrogen and acetyl percentages differ, although the strain is still positive to Inaba 'O' antiserum. That such changes occur emphasizes the necessity of using freshly isolated cholera strain in any study of antigenic structure. It would be of interest to study the possibility of regenerating the polysaccharide-lipid complex in old laboratory strains. It is quite possible that the observed changes in chemical constitution may have a bearing on the usefulness of strains in preparing vaccines.

The cross-reactions between the two types of strains from cholera cases is to be expected from a consideration of their polysaccharides. Both of these have the same aldobionic acid, although differing in having, in addition, galactose in one and arabinose in the other. The haptens are thus closely related although not identical. A further step in the study of these cross-reactions would include quantitative estimations of the amount of precipitin antibodies present in any particular antiserum by precipitating it with homologous polysaccharide and comparing it with the amounts precipitated with heterologous polysaccharide (Heidelberger, Kabat and Shrivastava, 1937). Recently, Lancefield (1938) has reported the presence of related but not identical type-specific substances in two closely related types of group B. hæmolytic streptococcus.

Our present serological results have further shown that polysaccharides of group II and group III organisms are different, although both of them contain the same aldobionic acid and arabinose. More detailed work is needed to determine the structure of these two more closely.

The water vibrios are less homogeneous than the group I and group II strains. This observation is similar to that of numerous workers, who have found much heterogeneity in this group.

In conclusion, we may point out various lines in the study of vibrio antigenic structure which require further study: (1) The function of the

lipoid-polysaccharide complex, with especial reference to its relationship to pathogenicity. (2) The possibility of producing the lipoid fraction in strains in which it is absent. (3) The application of the quantitative method to the precipitin reactions studied in this work, with a view to clearing up the cross-reactions, and putting them on a quantitative basis. (4) The proteins and their rôle in vibrio serology. For this study, protein fractions should be isolated by the recently developed methods used with other organisms.

SUMMARY.

1. Specific polysaccharides have been prepared from 23 strains of vibrios belonging to various chemical groups and their chemical and serological properties studied.

2. Precipitin reactions between the polysaccharides and antisera to the whole organisms indicate that in general the serology expresses the underlying chemical pattern of these organisms, and indicates the same groupings as the chemical analysis.

3. The presence of two chemically and serologically distinct types has been shown among the cholera strains. The chief point of difference is that the strains from the early part of the epidemic contain a lipoid-polysaccharide complex, which is absent in strains obtained from the latter part of the epidemic, in strains maintained for a long time in the laboratory, and in water and carrier strains. The probable importance of this complex has been emphasized.

4. Water vibrios and carrier strains have been shown to be heterogeneous among themselves.

5. The importance of using freshly isolated strains in the study of the antigenic structure of the vibrios has been emphasized.

6. Some lines along which this work might be continued are pointed out.

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TABLE I.

Strain.	Chemical group.	Yield per litre, mg.	Ash per cent.*	Specific rotation $[\alpha]_D^\dagger$	N ₂ , per cent.†	Acetyl group, per cent.†	Maximum hydrolysis, per cent.†	REMARKS.
202	I	1.9	..	+ 85.5	5.9	1.4	32.8	Marked opalescence on the addition of H ₂ SO ₄ to aqueous solution.
179	I	2.7	5.8	+ 74.9	4.7	1.3	46.1	Do.
196	I	2.6	7.8	+ 136.9	7.1	2.1	38.7	Do.
198	I and II	2.7	..	+ 80.6	5.5	0.0	42.8	Do.
176	II	1.9	7.8	+ 120.0	9.1	2.6	31.2	Do.
200	II	2.8	7.7	+ 82.7	8.0	1.3	34.7	Do.
334	II	3.8	5.8	+ 94.1	6.9	3.7	43.4	Do.
319	II	2.4	5.2	+ 84.5	3.1	2.8	59.0	Very slight opalescence.
322	II	4.0	5.4	+ 90.5	3.2	3.3	54.8	Do.
330	II	1.9	4.8	+ 95.2	2.4	4.7	63.4	Do.
315	II	4.0	4.6	+ 91.1	1.4	2.6	56.1	No opalescence.
Inaba + T	II	2.7	5.1	+ 83.2	1.8	6.3	55.9	Do.

* As Na₂O.

† Calculated on ash-free basis.

Hydrolysis was carried out in N/1 H₂SO₄ in sealed tubes in boiling water-bath. The estimations of reducing substances were made by the Hagedorn and Jensen method and the results are expressed in terms of glucose.

TABLE II.

Strain.	Chemical group.	Yield per litre, mg.	Ash, per cent.*	Specific rotation $[\alpha]_D^\dagger$	N ₂ , per cent.†	Acetyl group, per cent.†	Maximum hydrolysis, per cent.†	REMARKS.
W132	III	2.9	5.7	+ 43.8	2.7	..	49.1	No opalescence.
W138/2	III	4.2	6.0	+ 59.5	2.5	4.9	48.0	Do.
W139/2	III	3.7	6.5	+ 80.6	1.6	12.9	47.4	Do.
W164	III	9.6	6.6	+ 37.9	1.7	7.5	67.0	Do.
W182	III	10.5	6.1	+ 71.7	2.2	5.4	57.2	Do.
W171	III and IV	9.5	5.4	+ 96.4	1.5	5.0	67.2	Do.
W115/5	IV	3.7	5.0	+ 61.1	3.2	..	61.0	Do.
H5	IV	3.3	6.5	+ 42.4	3.1	5.4	48.6	Do.
H4	IV and V	1.5	..	+ 84.8	2.9	3.9	41.5	Do.
H25	IV and V	0.6	..	+ 87.5	68.6	Do.
1894	IV and V	10.2	4.4	+118.4	3.1	5.7	58.2	Do.
Inaba variant.	VI	16.4	5.9	+ 53.0	3.2	12.8	65.8	Do.

* As Na₂O.

† Calculated on ash-free basis.

Hydrolysis was carried out in N/1 H₂SO₄ in sealed tubes in boiling water-bath. The estimations of reducing substance were made by the Hagedorn and Jensen method and the results are expressed in terms of glucose.

TABLE III.

Strains.	Chemical groups.	Homologous titres.
202	I	2.0×10^5
179	I	2.5×10^5
196	I	2.5×10^5
198	I and II	2.0×10^6
176	II	2.5×10^5
334	II	2.5×10^5
319	II	2.0×10^5
322	II	2.0×10^5
330	II	6.4×10^4
315	II	2.0×10^5
Inaba 'T'	II	1.0×10^5
W132	III	5.0×10^5
W138/2	III	4.0×10^5
W139/2	III	6.4×10^4
W164	III	6.4×10^4
W182	III	1.0×10^5
W115/5	IV	4.0×10^5
H5	IV	5.0×10^5
H4	IV and V	2.5×10^5
H25	IV and V	2.5×10^5
1894	IV and V	5.0×10^5
Inaba variant.	VI	16.0×10^5

TABLE IV.

ANTISERA.

Polysaccharides.	ANTISERA.												H25 (IV and V).	1894 (IV and V).	Inaba variant (VI).
	202 (I)*	179 (I)	196 (I)	198 (I and II)	176 (II)	334 (II)	319 (II)	322 (II)	330 (II)	315 (II)	Inaba, 'T', (II).				
202 (I)	100	100	100	50	100	12.5	25	100	100	100	0	0	25	0	0
179 (I)	25	100	25	100	0	12.5	12.5	100	100	50	50	0	12.5	25	0
196 (I)	25	0	100	50	0	12.5	0	12.5	0	100	50	0	12.5	25	0
198 (I and II).	50	50	12.5	100	0	50	25	25	50	12.5	0	0	12.5	50	0
176 (II)	25	100	100	50	100	25	50	50	25	50	0	0	25	100	0
200 (II)	100	50	25	50	0	50	25	12.5	50	12.5	25	0	25	50	0
334 (II)	0	0	12.5	12.5	0	100	25	25	25	25	12.5	0	12.5	0	0
319 (II)	25	0	0	25	0	100	25	100	100	100	0	0	25	0	0
322 (II)	100	12.5	12.5	50	50	100	100	100	100	50	25	0	25	0	0
330 (II)	50	0	0	25	0	25	50	100	100	50	0	0	25	0	0
315 (II)	50	0	0	50	0	25	50	50	100	100	0	0	25	0	0
Inaba, 'T', (II).	25	12.5	0	50	50	50	25	0	25	25	100	0	12.5	0	0

* Figures within brackets indicate the chemical groups.

TABLE V.

Degree of cross-reaction, per cent.	PERCENTAGES OF THE TOTAL NUMBER OF CROSS-REACTIONS SET UP.			
	GROUP A.		GROUP B.	
	Homologous.	Heterologous against group B.	Homologous.	Heterologous against group A.
100	31.0	3.3	40.0	0.0
50	21.4	26.7	28.0	17.1
25	14.3	26.7	16.0	28.6
12.5	14.3	10.0	0.0	22.9
0	19.0	33.3	16.0	31.4

TABLE VI.

Polysaccharides.	ANTISERA.																
	202 (I)*	179 (I)	196 (I)	198 (I and II)	176 (II)	334 (II)	319 (II)	322 (II)	330 (II)	316 (II)	Inaba, T. (II)	W132 (III)	W138/2 (III)	W139/2 (III)	W164 (III)	W182 (III)	W115/5 (IV)
W132 (III)	0	0	0	0	0	0	0	0	0	0	0	100	0	0	25	0	0
W138/2 (III)	0	0	0	0	0	0	0	0	0	0	0	0	100	0	0	0	0
W139/2 (III)	0	0	0	0	0	0	0	0	0	0	0	0	0	100	0	0	0
W164 (III)	0	0	0	0	0	0	0	0	0	0	0	12.5	12.5	12.5	100	0	0
W182 (III)	0	0	0	0	0	0	0	0	0	0	0	0	100	12.5	0	0	0
W171 (III and IV).	0	0	0	0	0	0	0	0	0	0	0	0	12.5	12.5	0	0	0
W115/5 (IV)	25	0	0	0	0	50	0	0	0	0	0	0	0	0	0	100	0
H5 (IV)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100	0	0
H4 (IV)	12.5	0	0	0	0	25	0	0	12.5	12.5	0	0	0	0	100	25	12.5
H25 (IV and V).	50	12.5	12.5	0	25	0	0	0	0	0	0	0	0	12.5	50	100	0
1894 (IV and V).	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100	0
Inaba variant (VI).	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100

* Figures within brackets indicate the chemical groups.

THE DETECTION OF MALNUTRITION BY MEASUREMENTS OF ARM, CHEST, AND HIP.

BY

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IN an earlier paper (Aykroyd and Rajagopal, 1936) an investigation of the state of nutrition of school children in South India was reported. It was observed that certain symptoms associated with diet deficiency—angular stomatitis, Bitôt's spots (xerophthalmia), and 'phrynoderma' (follicular keratosis of the skin) were common in the children examined. The American A. C. H. index of nutrition, based on measurements of arm, chest, and hip, was applied to the same group, and it was found that some 25 per cent was 'selected', whereas the index is said to 'select' only some 10 per cent of groups of average American children. Now it is to be observed that the term 'selected', as applied to Indian children, may mean precisely nothing. We have taken a system of measurements based on American children and used it on children of a different race, and it cannot be assumed that a system which purports to pick out malnourished children in Massachusetts will do the same in Madras. To throw light on this question, the association between 'selection' by the index and the presence of deficiency disease was investigated. The following figures summarize the results :—

Number of children investigated.	Number and per cent selected by the A. C. H. index.	Number and per cent showing clinical signs.	Per cent of those showing signs selected.	Number <i>not</i> showing clinical signs.	Per cent of those <i>not</i> showing signs selected.
1,145 921 boys 224 girls	294 (25.1 per cent)	235 (20.5 per cent)	63.8	910 (79.5 per cent)	15.8

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The index 'selected' 63·8 per cent of children with signs as compared with 15·8 of children without signs. It was, therefore, evident that the index, although it is based on the measurements of American children and appears at first sight unlikely, for a number of reasons, to be applicable to Indian children, does tend to 'select' South Indian children who are suffering from malnutrition evidenced by signs of deficiency disease.

This was the starting point of the present investigation. We felt that the above figures were interesting and remarkable, and that the whole question was worth further study. The present paper describes further investigations of the association between arm, chest, and hip measurements and deficiency disease, and of the question whether the A. C. H. index is applicable in India.

THE PROBLEM OUTLINED.

Some preliminary discussion of the problem is desirable. Many numerical measures or indices for assessing 'state of nutrition', or what amounts to very much the same thing, 'detecting malnutrition', have been devised. There is a general consensus of opinion that in practice these have all proved unsatisfactory. In the U. S. A. it is customary to assess a child's state of nutrition by comparing its 'weight for height' with standards based on the measurement of large numbers of healthy children. A child who falls 7 or 10 per cent below the standard is described as suffering from 'malnutrition'. A growing realization of the fallacies inherent in this practice led Franzen (1929) and Franzen and Palmer (1934), working on behalf of the American Child Health Association, to attempt a new approach to the problem. The A. C. H. index (A = arm, C = chest, H = hip) is the result of their work. Its rationale is as follows: A large series of children (over 10,000) was examined and their state of nutrition assessed clinically by experienced pædiatricians. In the same children a very complete set of measurements was taken, including height, weight, shoulder breadth, hip width, chest width and depth, arm and calf girth, and thickness of the subcutaneous tissue in different parts of the arms and legs. By statistical treatment the correlation between clinical malnutrition and the various measurements was worked out. Ultimately it was found that a certain relationship between arm, chest, and hip measurements was associated with a diagnosis of malnutrition, other measurements being discarded as unnecessary for most practical purposes. In practice the observer measures the circumference of the right arm at the highest point of the biceps with the forearm flexed and extended*; the anterior-posterior diameter of the chest during inspiration and expiration; and the width of the hips from trochanter to trochanter. He then consults a chart which gives 'the minimum difference between arm girth and chest girth' for the known hip width of the child. If the difference between the sum of the two arm measurements and the sum of the two chest measurements is less than the figure on the chart, the child is 'selected' as suffering from malnutrition. The index, then, purports to pick out children to whom an experienced physician would affix the label 'malnutrition'. Such children are said to have 'small amounts of musculature and fatty tissue relative to body-build'. Statistical analysis revealed that American physicians, in assessing

* We have used the term 'extended' in preference to Franzen and Palmer's term 'relaxed'. 'Extended' in the present paper means extended in the relaxed position, i.e., hanging.

state of nutrition, pay more attention to the state of muscular and adipose tissue than to any other signs or symptom. We shall, however, show later that the A. C. H. system is in fact concerned less with the amount of muscle and fat relative to body-build than with other bodily changes associated with malnutrition.

The original A. C. H. chart is given in Table I. It is stated to apply to the age group 7 to 12. The following is an example of the method of using it: Suppose a boy's hip width is between 23.0 cm. and 23.4 cm. If the difference between arm girth and chest depth is less than 3.3 cm., he is 'selected'.

TABLE I.

The A. C. H. chart.

(American criterion table to select 10 per cent.)

Boys.		Girls.	
Width of hips (cm.).	Minimum difference between arm girth and chest depth (cm.).	Width of hips (cm.).	Minimum difference between arm girth and chest depth (cm.).
Below 20.0	0.0	Below 20.0	0.5
20.0-20.0	0.0	20.0-20.4	1.5
20.5-20.9	0.4	20.5-20.9	1.6
21.0-21.4	1.0	21.0-21.4	2.1
21.5-21.9	1.6	21.5-21.9	2.6
22.0-22.4	2.2	22.0-22.4	3.0
22.5-22.9	2.7	22.5-22.9	3.4
23.0-23.4	3.3	23.0-23.4	3.8
23.5-23.9	3.6	23.5-23.9	4.2
24.0-24.4	4.2	24.0-24.4	4.5
24.5-24.9	4.7	24.5-24.9	4.8
25.0-25.4	5.1	25.0-25.4	5.1
25.5-25.9	5.6	25.5-25.9	5.4
26.0-26.4	6.0	26.0-26.4	5.6
26.5-26.9	6.3	26.5-26.9	5.8
27.0-27.4	6.7	27.0-27.4	6.0
27.5-27.9	7.0	27.5-27.9	6.1
28.0-28.4	7.3	28.0-28.4	6.2
28.5-28.9	7.6	28.5-28.9	6.3
29.0 and over.	7.9	29.0 and over.	6.4

In the construction of the original index the diagnosis of malnutrition was made by general clinical assessment. In the present work a different criterion was used, namely, the presence of clinical signs of deficiency disease. Let us assume that long-continued malnutrition, caused by defective diet, will tend to produce changes in the structure of the body which can be recorded by external

60 *Detection of Malnutrition by Measurements of Arm, Chest, and Hip.*

In the earlier paper (Aykroyd and Rajagopal, *loc. cit.*) we recorded an impression that in general the state of nutrition of girls was better than that of boys. This was certainly the case in some schools. But an analysis of the sex incidence of signs in the first series does not substantiate this impression.

		Per cent.
Of 3,232 boys	514 showed signs	= 15.9
„ 510 girls	72 „ „	= 14.12

The smaller incidence among the girls is not statistically significant.

These figures in themselves illustrate the wretched state of nutrition of poor South Indian children.

The A. C. H. index.—23.9 and 24.4 per cent were ‘selected’ in the first and second series respectively. The association between ‘selection’ and deficiency disease was observed throughout, as the following figures show:—

	Number of children.	Number and per cent ‘selected’.	Number and per cent showing signs.	Per cent of those showing signs selected.	Number and per cent <i>not</i> showing clinical signs.	Per cent of those <i>not</i> showing signs selected.
First series ..	3,742 3,232 boys 510 girls	887 (23.7 per cent)	586 (15.7 per cent)	75.7	3,156 (84.1 per cent)	14.0
Second series ..	904 boys	221 (24.4 per cent)	153 (16.9 per cent)	80.4	751 (83.1 per cent)	13.5
TOTAL ..	4,646	23.8 per cent.	15.9 per cent.	76.7 per cent.	84.1 per cent.	13.9 per cent.

STATISTICAL ANALYSIS.*

Material.

The data for analysis consisted of records of the examination of 3,232 boys and 510 girls (first series) and 904 boys (second series). These records were made on cards (one for each child) and included the following: (1) Age. (2) Arm girth (a) flexed and (b) extended, and (c) the total of these two measurements. (3) The anterior-posterior diameter of the chest at (a) expiration and (b) inspiration, and (c) the total of these two measurements. (4) Hip width. (5) Difference between totals of (2) and (3). The presence of one or more of the three signs—angular stomatitis, Bitôt’s spots, and phrynoderma—was indicated by inserting a plus mark on the card against the names of the signs.

* Carried out by K. B. Madhava.

Age.

Age, as entered in school records, was noted in all cases. In the tables which follow all children whose ages were recorded as 10 years and so many months were placed in age group 10-11, and the same for other ages. The mean age of this group is assumed to be 10·5 years, and the attributes ascribed to 10·5 years should be taken as relating to this age group. The lowest age recorded was 5, and highest 16, but in the range 6 to 14 more than 99 per cent of the children were included, while all but the extreme 19 per cent fell between 7 and 12. The mean ages for children 'affected' and 'free', with related statistical constants, are given in Table II. The actual frequency distributions for each year of 'free' and 'affected' children (first and second series) are given in Table A of the *Appendix*.

TABLE II.

Mean age with statistical constants.

		Mean and standard error.	Standard deviation and standard error.
<i>First series.</i>			
Boys	(1) Affected ..	9·89±0·08	1·85±0·06
	(2) Free ..	9·44±0·04	1·83±0·03
Girls	(1) Affected ..	9·14±0·24	2·02±0·17
	(2) Free ..	9·05±0·09	1·93±0·06
<i>Second series.</i>			
Boys	(1) Affected ..	9·37±0·13	1·57±0·09
	(2) Free ..	8·96±0·06	1·59±0·04

It will be noted that on the average the boys in the second series were about half a year younger than those in the first series; their ages were also less variable, which is probably due to the fact that they were attending a homogeneous group of schools in Madras. The average age of the girls is similarly lower than that of the boys in the first series; a lower average age for girls is a characteristic of the school-going population in India. More striking is the higher average age of the 'affected' children as compared with that of the 'free' children. This might be taken to imply that the clinical signs looked for are more common in, or perhaps more prominently visible in, senior children, but the difference in the means are not statistically significant except in the case of the boys in the first series. It is to be observed that the recorded age of school children of the poorer classes in India is subject to a high margin of error, but presumably the effect of such errors would be distributed constantly throughout the groups.

Arm measurements.

Arm measurements recorded consisted of the circumference of the arm (a) in the flexed position, (b) in the extended position, and (c) the total of these measurements. In Tables B, C, and D (*Appendix*) frequency distributions are given by ranges of one cm. The mean value of the measurements of all the children included in a given range is the middle point of the range. The means with standard error and standard deviation with standard error are given in Table III:—

TABLE III.

Mean arm girths with statistical constants.

			MEAN WITH STANDARD ERROR.			STANDARD DEVIATION WITH STANDARD ERROR.		
			Flexed.	Extended.	Total.	Flexed.	Extended.	Total.
<i>First series.</i>								
Boys	(1) Affected	..	14.89	14.11	28.66	1.68	1.57	3.33
			± 0.07	± 0.07	± 0.15	± 0.05	± 0.05	± 0.11
	(2) Free	..	14.97	14.09	28.77	1.54	1.44	2.98
			± 0.03	± 0.03	± 0.06	± 0.02	± 0.02	± 0.04
Girls	(1) Affected	..	15.57	14.68	30.08	1.59	1.42	3.04
			± 0.19	± 0.17	± 0.36	± 0.14	± 0.12	± 0.25
	(2) Free	..	15.50	14.64	29.93	1.85	1.59	3.22
			± 0.09	± 0.08	± 0.36	± 0.06	± 0.06	± 0.11
<i>Second series.</i>								
Boys	(1) Affected	..	14.11	13.47	27.17	1.26	1.18	2.45
			± 0.10	± 0.10	± 0.20	± 0.07	± 0.07	± 0.14
	(2) Free	..	14.59	13.82	28.10	1.39	1.25	2.63
			± 0.05	± 0.05	± 0.10	± 0.04	± 0.03	± 0.07

The points that attract attention in Table III are, firstly, that the boys in the second series have smaller mean arm girths than those in the first series. This difference is apparent whether arm girth in the flexed position, or the extended position, or the total of these measures, is considered. It can probably be to some extent accounted for by the smaller mean age of the second series. The girths in the second series are also less variable than in the first series. Secondly, the girls show larger arm girths than the boys, in spite of the fact that their mean age is lower. The girls show differences in respect of other measurements to be considered later. Their superiority in arm girth may perhaps be ascribed to the fact

that in India girls attending school tend to be drawn from a socially superior class of families, or it may simply be due to feminine body-build. Thirdly, it is important to note (since we are here considering measurements which single out children showing signs from those not so affected) that the 'affected' children, as compared with the 'free' children, do not show consistently larger or smaller mean values. None of the observed differences in the means given in Table III are statistically significant.

Chest measurements.

The statistical constants relating to the chest measurements are given in Table IV and the frequency distributions in Tables E, F, and G in the *Appendix*.

TABLE IV.

Mean chest measurements with statistical constants.

		MEAN AND STANDARD ERROR.			STANDARD DEVIATION AND STANDARD ERROR.		
		Expira- tion.	Inspira- tion.	Total.	Expira- tion.	Inspira- tion.	Total.
<i>First series.</i>							
Boys	(1) Affected ..	14.31	14.75	28.67	1.15	1.20	2.34
		± 0.05	± 0.05	± 0.10	± 0.04	± 0.04	± 0.07
	(2) Free ..	12.99	13.43	26.01	1.19	1.21	2.39
		± 0.03	± 0.02	± 0.05	± 0.05	± 0.02	± 0.03
Girls	(1) Affected ..	13.39	14.43	27.90	1.19	1.11	2.26
		± 0.14	± 0.13	± 0.27	± 0.10	± 0.09	± 0.19
	(2) Free ..	13.17	13.55	26.32	1.18	1.16	2.35
		± 0.06	± 0.06	± 0.11	± 0.04	± 0.04	± 0.08
<i>Second series.</i>							
Boys	(1) Affected ..	13.98	14.51	28.09	0.96	1.00	1.85
		± 0.05	± 0.05	± 0.10	± 0.04	± 0.04	± 0.07
	(2) Free ..	12.73	13.26	25.60	0.85	0.88	1.66
		± 0.02	± 0.02	± 0.04	± 0.01	± 0.02	± 0.03

Table IV shows, like the previous ones, smaller variability of the measures in the second series. The means are smaller in the second series, but the differences between these and those calculated for the first series cease to persist when

correction is made for the smaller mean age in the second series. In contradistinction to the arm girth measurements, the chest measurements in the girls do not exceed those of the boys, being in fact actually smaller.

The most important point brought out in Table IV is the *increased mean measurements* in the groups of 'affected' children as compared with the groups of 'free' children. Some part of the difference is ascribable to the difference in the mean ages, but even after this correction is made the chest measurements remain significantly larger in the affected group. This excess, taken with the deficit in arm girth in the affected group noted in the last section, gives rise to the disparity between arm girth and chest depth which forms the basis of the A. C. H. index.

Disparity between arm and chest measurements (G).

The difference between the total of the two arm girth measurements and that of the two chest measurements is a character of fundamental importance in the A. C. H. system. In the present paper we call this character G.

$G = \text{arm girth with the forearm flexed plus arm girth with the forearm extended minus chest depth during inspiration plus chest depth during expiration.}$ —In the following pages G will be given a negative sign if the sum of the former pair of measurements exceeds the sum of the latter pair.

The statistical constants obtained for G are given in Table V:—

TABLE V.

Mean for character G with statistical constants.

			Mean with standard error.	Standard deviation, with standard error.
<i>First series.</i>				
Boys	(1) Affected	..	0.25 ± 0.10	2.27 ± 0.07
	(2) Free	..	2.65 ± 0.03	1.79 ± 0.02
Girls	(1) Affected	..	1.93 ± 0.29	2.48 ± 0.20
	(2) Free	..	3.52 ± 0.10	2.06 ± 0.07
<i>Second series.</i>				
Boys	(1) Affected	..	1.17 ± 0.12	2.28 ± 0.09
	(2) Free	..	2.40 ± 0.05	1.95 ± 0.03

The frequency distribution of G is given in Table H in the *Appendix*. It will be noted that in each group the affected children have smaller values of G, the distribution tables for these commencing with larger negative values than those exhibited by the 'free' group and tending to terminate before large positive values

are reached. The fractions in each group of children possessing negative values of G are as follows:—

				Affected.	Free.
				Per cent.	Per cent.
First series	Boys	59	5
	Girls	24	2
Second series.	Boys	72	9
				—	—
TOTAL				58	5
				—	—

Thus, a child with signs is more likely to show a negative than a positive value of G (nearly 60 per cent in the 'affected' group had such a value), whereas such a value appears in only about 1 in 20 of the 'free' group.

These results show definitely that a low value of G tends to occur in the children showing signs. It is obvious that this character may be of great importance in connection with the problem of assessing the 'state of nutrition' of children.

Width of hips.

The second character which is prominent in the A. C. H. system is hip width. Means with statistical constants are given in Table VI, and the frequency distribution in Table I in the *Appendix*.

TABLE VI.

Mean hip width with statistical constants.

				Mean with standard error.	Standard deviation with standard error.
<i>First series.</i>					
Boys	{ (1) Affected	21.04 ± 0.08	1.81 ± 0.06
	{ (2) Free	20.29 ± 0.03	1.64 ± 0.02
Girls	{ (1) Affected	21.22 ± 0.24	2.03 ± 0.17
	{ (2) Free	20.52 ± 0.10	2.06 ± 0.07
<i>Second series.</i>					
Boys	{ (1) Affected	20.99 ± 0.07	1.28 ± 0.05
	{ (2) Free	20.28 ± 0.03	1.35 ± 0.02

As before, there was less variability in the second series, though the mean hip width in this series does not differ from that of the boys in the first series. There is, also as before, a tendency for the mean to be higher in girls, but not to an extent permitting statistical significance. It is interesting to note that, while the 'affected' group showed smaller G measurements, their mean hip measurements are actually larger (even when allowance is made for the slightly greater mean age of these groups). This fact throws into relief the relatively small

values of G which occur in the 'affected' group in association with mean values for other measurements which are similar to, or larger than, those exhibited by the 'free' groups.

The character G^1 .

To obtain the character G , two measurements of arm girth and chest diameter, respectively, are made. The question arises whether it is feasible to eliminate one measure in each case. This would be advantageous, since it would reduce the labour of examination provided that the two single measures were equally efficacious. The two measures of arm and chest relate to almost the same trait and are obviously directly connected with each other. The correlation co-efficients between arm girth measured in the flexed and extended positions are high, being as follows:—

				<i>Free.</i>	<i>Affected.</i>
First series	Boys	0.97	0.96
	Girls	0.96	0.97
Second series	Boys	0.95	0.92

The probable error is less than 0.01. The standard deviations of the measures are also alike. Consequently, if one of these measures is determined, the other, which varies from it in simple proportion, can be ascertained by simple linear relation. There is, therefore, justification for eliminating one of the measures. Similar arguments can be put forward in the case of the two chest diameter measurements, which are correlated in the same order.

It is of interest to determine the relation between the presence and absence of signs and the difference between a single arm and a single chest measurement, and compare it with that observed when four measures are employed. Accordingly arm girth in extension and chest diameter at inspiration was eliminated, and the remaining two measures used in the same way as the total of the two measures, i.e., for measuring the excess of arm girth over chest depth. This new character (b—d) we have called G^1 .

TABLE VII.

Mean values of G^1 with statistical constants.

				Mean with standard error.	Standard deviation with standard error.
<i>First series.</i>					
Boys	{ (1) Affected	..		0.70 ± 0.04	1.22 ± 0.03
	{ (2) Free	..		2.15 ± 0.01	0.99 ± 0.01
Girls	{ (1) Affected	..		1.82 ± 0.15	1.25 ± 0.10
	{ (2) Free	..		2.57 ± 0.05	1.11 ± 0.04
<i>Second series.</i>					
Boys	{ (1) Affected	..		0.14	1.26
	{ (2) Free		1.96	1.05

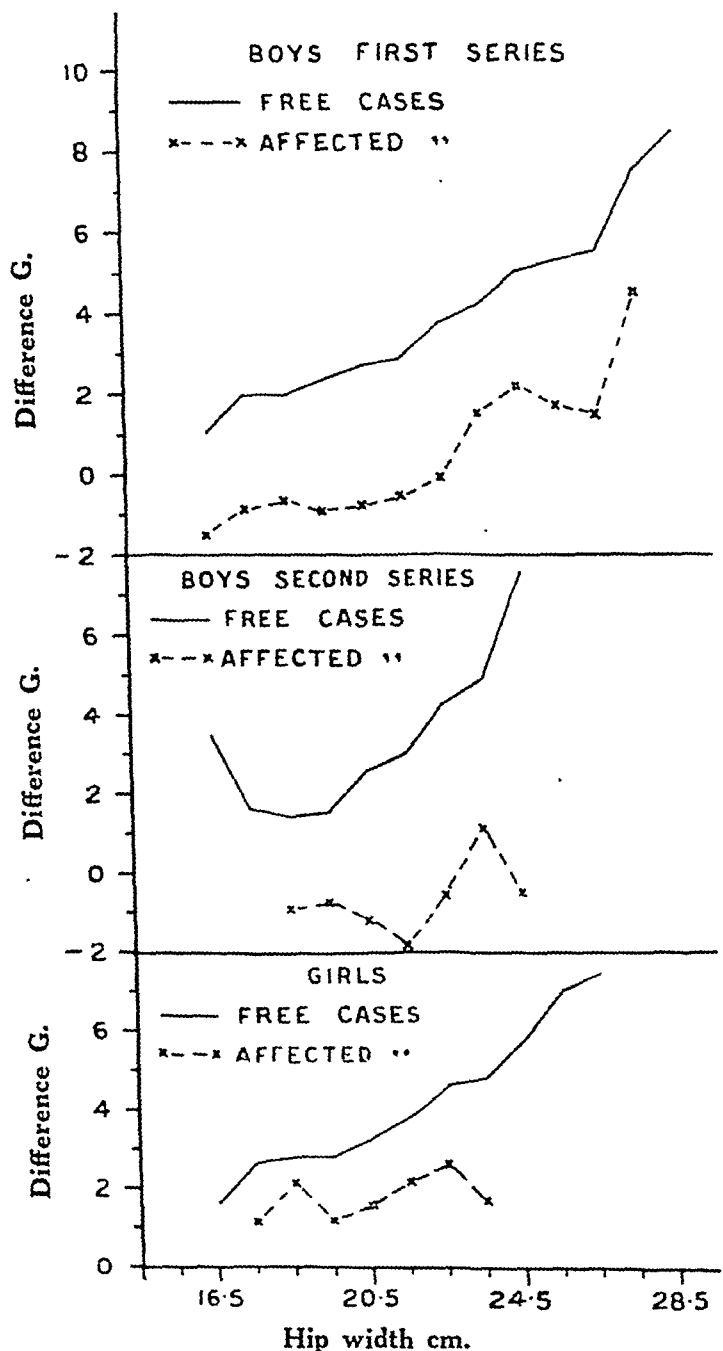


CHART A.—Values of G (sum of arm measurements minus sum of chest measurements) for different values of hip width in free and affected groups.

It is noteworthy that G^1 possesses the same characteristics as G . The frequency distribution (Table J, *Appendix*) shows that negative values of G^1 are preponderant in the 'affected' children in much the same degree as negative values of G . Mean statistical constants are given in Table VII. These indicate a similar association between high and low mean values of G^1 and the presence and absence of signs as was observed in the case of G .

Charts A and B show mean values for G and G^1 for a given hip width in the free and affected groups. It is apparent that the two groups are as clearly differentiated by the relation of G^1 to hip width as by the relation of G to the same measure.

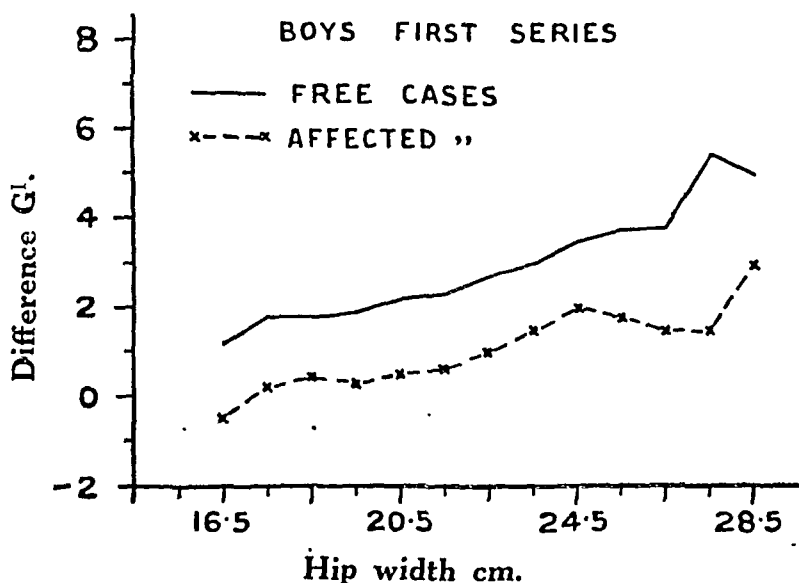


CHART B.— G^1 (arm girth flexed *minus* chest depth at expiration) for different values of hip width in free and affected groups.

The relation of age to arm girth, chest depth, hip width, and G .

The relation of the various measures to age will now be considered. The necessity of introducing corrections to offset age differences in the various groups has been pointed out in earlier sections. In order to study this question further the association of the various characters with age has been studied by correlational analysis. The correlation co-efficients obtained are given in Table VIII.

The correlation between age and all the direct body measurements is fairly high, being of the order 0.50 for arm girth and chest depth and a little higher 0.60 for hip width. The co-efficient is small in the case of G which, it will be noted, is not a body measurement, but a character derived by subtracting one set of measurements from another.

TABLE VIII.

Correlation co-efficients between age and various characters.

	AFFECTED.			FREE.		
	Boys.		GIRLS.	Boys.		GIRLS.
	1st survey.	2nd survey.		1st survey.	2nd survey.	
Number examined ..	514	153	72	2,718	751	438
Age with arm girth (flexed)	0.48	0.50	0.55	0.53	0.31	0.60
" " " " (extended)	0.49	0.52	0.57	0.53	0.50	0.60
" " " " total ..	0.48	0.50	0.57	0.53	0.51	0.60
" " chest depth :						
" " " " inspiration	0.44	0.38	0.48	0.48	0.40	0.56
" " " " expiration	0.46	0.40	0.51	0.48	0.38	0.55
" " total ..	0.46	0.42	0.50	0.49	0.42	0.54
G	0.24	0.12	0.27	0.23	0.34	0.34
Hip width	0.61	0.52	0.70	0.64	0.61	0.72
G ¹	0.70	0.63				

The measurements naturally increase with age. This is shown diagrammatically in Charts C, D, and E.

In Chart C the curve showing the sum of the two arm girth measurements at different ages for the 'affected' and 'free' cases run close to each other, intersecting fairly frequently; this illustrates the fact previously pointed out in connection with the mean values, that the 'free' and 'affected' groups are not really distinguishable in respect of the measurement. In the case of the boys, any advantage in size of arm girth appears to rest with the 'free' cases. The difference between the age groups in respect of the sum of the two chest measurements (Chart D) is more marked: here the advantage as regards size is definitely in favour of the 'affected' groups. As regards hip width (Chart E), the 'affected' and 'free' groups again show a slight but definite differentiation, the 'affected' curve being above the 'free' curve.

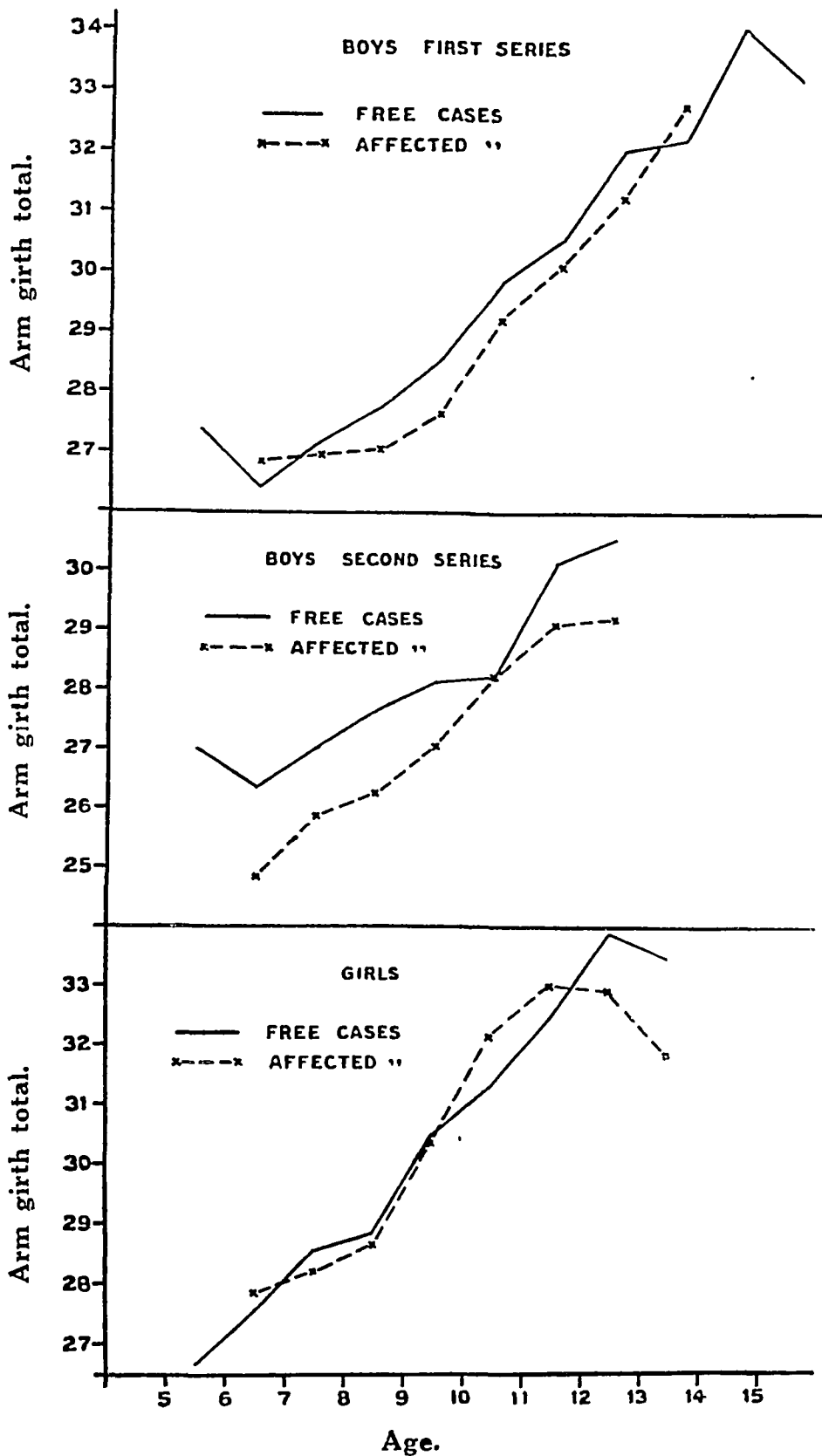


CHART C.—Total of two arm girth measurements (cm.) in the free and affected groups at various ages.

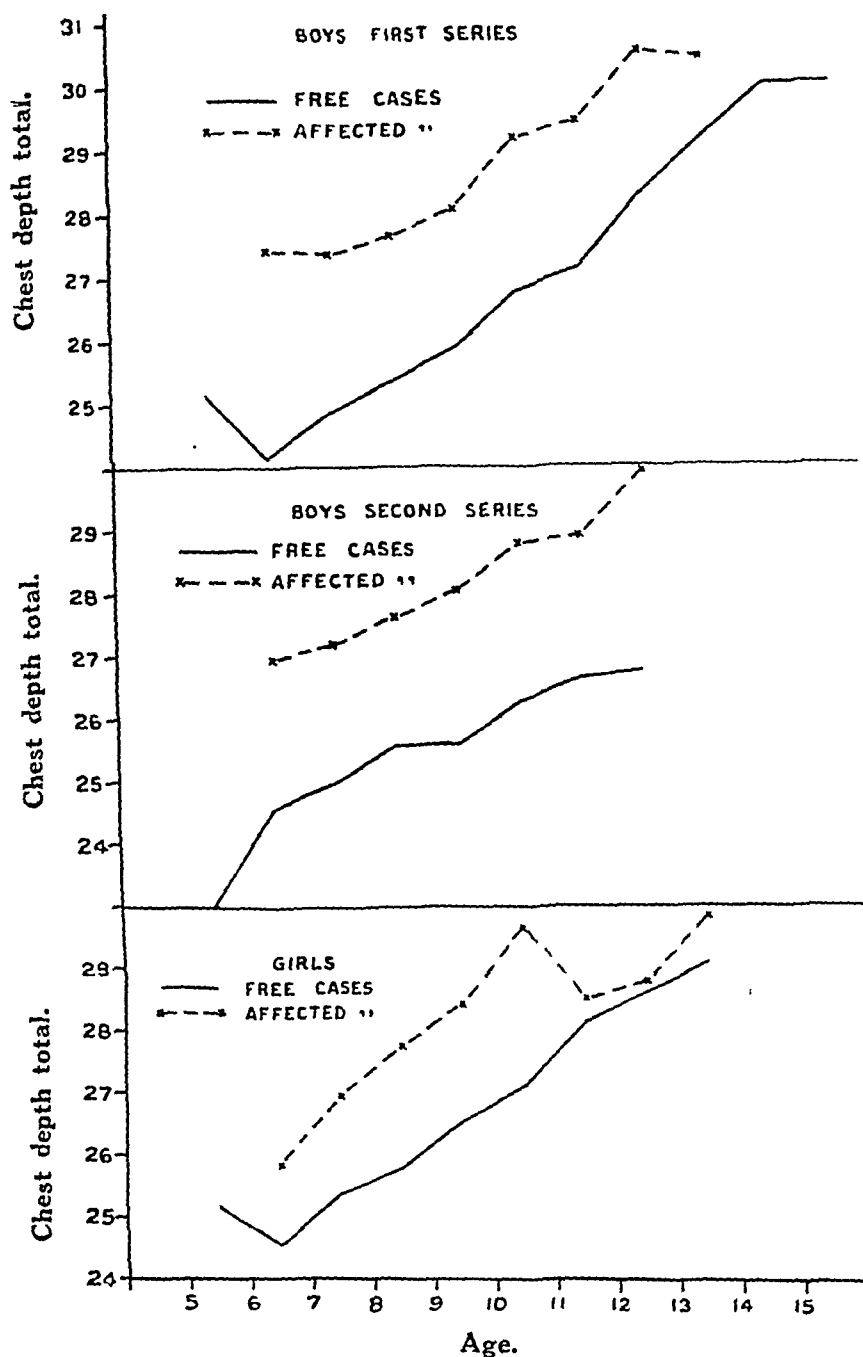


CHART D.—Total of chest depth measurements (cm.) in the free and affected groups at various ages.

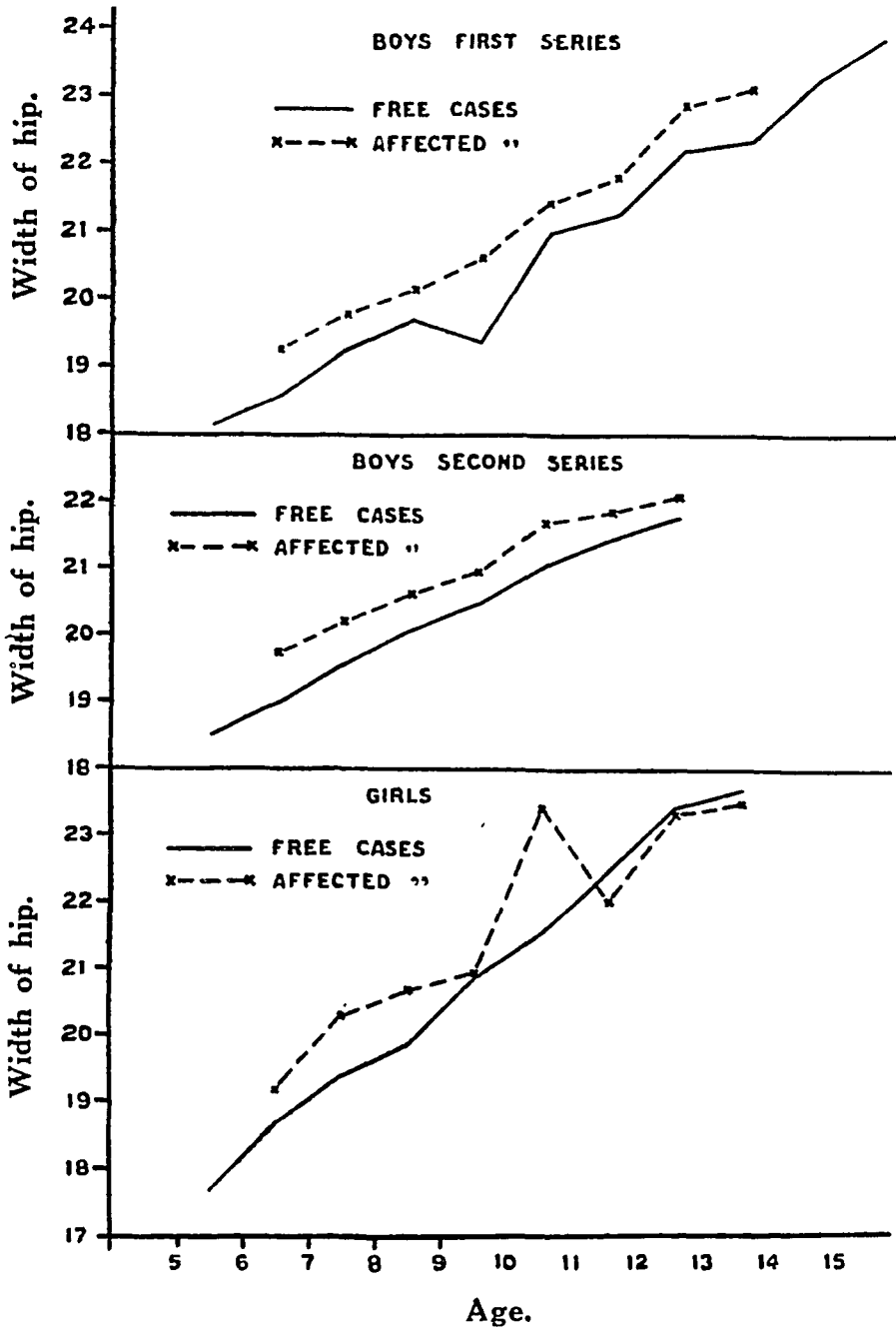


CHART E.—Hip width (cm.) in the free and affected groups at various ages.

The curves shown in Chart F, in which mean values of G for the various age groups are plotted, are very striking. They show how clearly the two groups are differentiated as regards the character G, the 'free' groups having considerably higher values in all age groups. At a constant age the two groups are situated quite differently in respect to the development of the arms in relation to that of the chest.

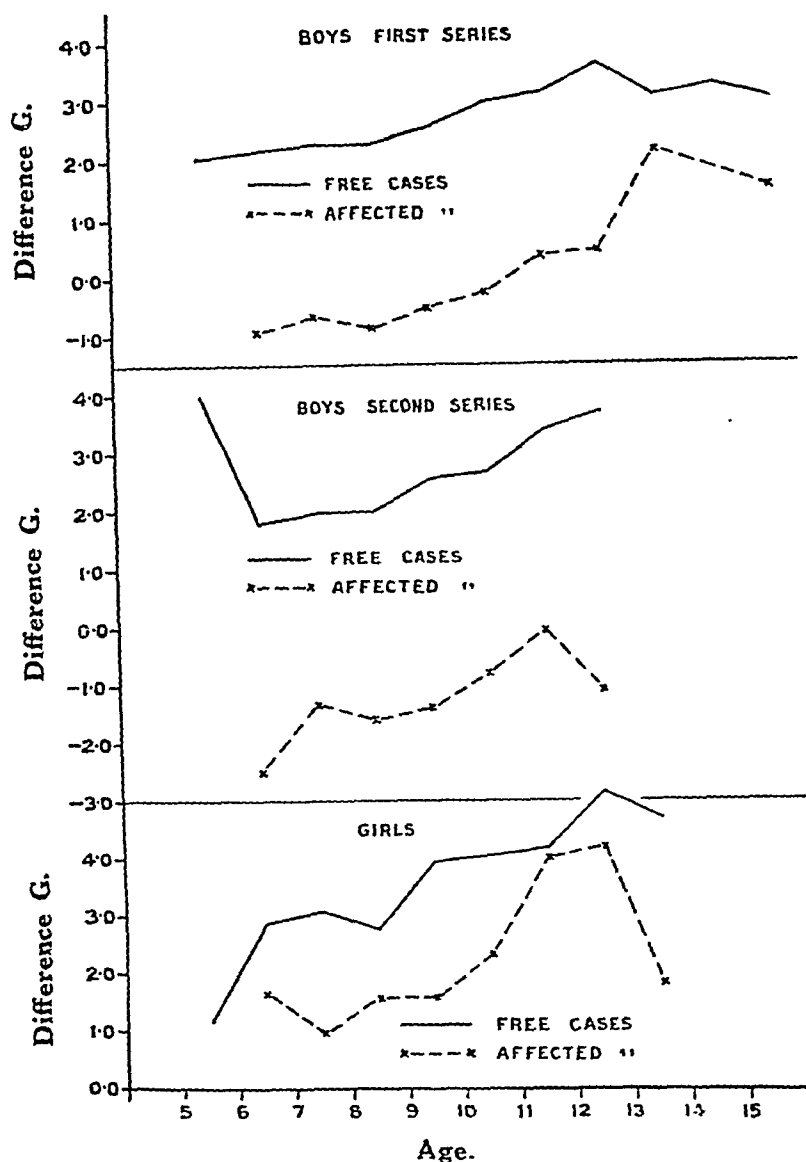


CHART F.—Values of G in free and affected groups at various ages.

The association between selection and the presence of signs is very clearly demonstrated in Charts G and H, in which values of G for free and affected children in two schools are plotted against hip width, and the A. C. H. criterion table is included in graphical form. In school A (Chart G) the cleavage of the two groups by the A. C. H. line is strikingly clear-cut particularly if the line is extended to

DISCUSSION.

A number of interesting questions arise out of these investigations. First, it is clear that the A. C. H. system, as a method of detecting malnutrition, merits greater attention than it has received from those concerned with the routine examination of school children. The index definitely 'selects' a high percentage of children suffering from severe malnutrition as shown by the presence of signs of deficiency disease. Since existing methods of assessing 'state of nutrition' or detecting malnutrition are generally agreed to be unsatisfactory, any new method should be given close study.

It appears that the American pædiatricians who collaborated in the establishment of the index did really succeed in making a reasonably satisfactory assessment of 'state of nutrition' on the basis of a general clinical examination. The index, based on their clinical assessment, can select a high percentage of children assessed as malnourished by a quite different criterion in another continent. Doubts are frequently expressed whether the clinical assessment of state of nutrition is of any value at all, and the discordant results obtained by officers of the school medical service in Great Britain lend weight to this criticism. Owing to the subjective element, widely different figures for the percentage malnutrition rate may be returned from essentially similar districts. The present investigation shows indirectly that a clinical observer can detect 'malnutrition' from a general examination. The observers concerned were, however, described as 'experienced pædiatricians' and a very careful and detailed clinical examination was made. This may be the crux of the matter. An index based on less exacting examinations carried out by less experienced observers would probably show a much smaller degree of correlation with a different clinical criterion of malnutrition.

Franzen and Palmer (*loc. cit.*) state that the A. C. H. index identifies children 'with small amounts of musculature and fatty tissue relative to body-build' and express the view that this is the most important criterion in determining the state of nutrition of school children. But deeper consideration suggests that an *increased anterior-posterior diameter of the chest* is the fundamental character on which the index is based. Table IV and Chart D show the difference in average chest measurements in the two groups. In Chart J chest depth in the two groups (school A) is plotted against age. It is obvious that a criterion table based on deviation from average chest depth would, in this particular school, 'select' children having signs almost as effectively as the A. C. H. index itself (compare Chart G).

There may be a relation between the A. C. H. index and 'the Sign of the Dorsal Median Furrow' described by the Norwegian workers Knudsen and Schiøtz. A description of this method of detecting malnutrition is given by Bigwood (1937) in his review on 'Methods of assessing the state of nutrition and adolescents considered in relation to defective diet'. The following is a quotation from the review:—

'One sign specially recommended by Schiøtz in mass investigations, as constituting the true state of nutrition, is the appearance of the median furrow of the back, when the arms are held straight above the head. The observer should look out for two points: the appearance of the spinal column viewed from behind

and the posture of the individual examined in profile. This test was discovered first by Knudsen in Denmark. It is claimed to be an indication of defective nutrition when the median furrow is broken or unduly sinuous, the former sign being the more important to watch for. According to the experience so far gained at Oslo, this is a simple and accurate test for the state of nutrition and is very little influenced by corrective gymnastics. The sign appears to depend really on the normal development of the spine and thorax under the influence of a properly balanced diet. Professor Schiötz lays great stress on the advantages of this test in mass investigations, its facility of application and its reliability'.

A 'broken or unduly sinuous median furrow' may be associated with an increased anterior-posterior diameter of the chest.

Royster (1927) in his book 'Nutrition and Development' remarks that 'there is no single part of the body which is better index of development or the state of nutrition than the size and contour of the chest'. The present investigation in general supports this statement. It is noteworthy that rickets in South India is extremely rare, and the increased anterior-posterior diameter of the chest observed in the children with clinical signs of deficiency disease is, therefore, not essentially a rachitic deformity.

THE USE OF AN INDEX OF THE A. C. H. TYPE FOR PRACTICAL NUTRITION WORK IN INDIA.

One of the purposes of the present investigation was to discover whether the American A. C. H. index, or some suitable modification of it, could be used in the surveys of diet and 'state of nutrition' which are now being carried out in various parts of India, and whether the method could be recommended for school medical inspection. The original index was intended by Franzen and Palmer 'to be an aid in sorting out children who should be seen by the physician'. An index of nutrition can, however, equally well be used to determine percentage rates of malnutrition in groups of children. The research programme of the Laboratories includes surveys of the diet and the state of nutrition in various parts of India, and a method which would enable comparisons of state of nutrition in various parts of the country to be drawn on a strictly uniform basis would be very useful.

We feel that the A. C. H. method can be recommended as a useful supplementary method for assessing the state of nutrition of an individual child and also for determining the incidence of malnutrition in groups of children between 6 and 13. Our own data relate only to South India and it is possible that variations in body-build may render an index of this type inapplicable in other parts of India. In Calcutta, Wilson, Ahmad and Mitra (1937) found no positive correlation between selection by the index and height, weight, and economic status. On the other hand, the same workers observed in the Punjab an association between selection and the clinical assessment of state of nutrition and also between selection and a low intake of milk. Clearly the collection of data on a wider scale is necessary.

The groups studied in these investigations showed a high percentage of clinical signs of deficiency disease. In practice the determining of the incidence of signs

has proved a valuable and simple method of comparing nutrition in schools (Aykroyd and Krishnan, 1936). This method has also been advocated by Nicholls (1934) in Ceylon. But there may be malnourished groups in which the diet, while generally deficient, is not extremely deficient in the food factors lack of which gives rise to signs. A correlation between a system of body measurements and a clinical criterion of malnutrition having been established, as in the present investigation, it seems justifiable to use the same system alone to assess state of nutrition.

THE MODIFICATION OF THE AMERICAN A. C. H. INDEX.

With regard to the establishment of an index of the A. C. H. type especially adapted to Indian conditions, it appears that, if we are seeking a *single* index or criterion table, based on values of G and hip width, to separate 'free' from 'affected' cases, no great improvement on the American index is possible. A study of Charts G and H suggests that a line based on higher or lower values of G would affect a less satisfactory differentiation. The extension of the A. C. H. index to include negative values of G and hip width measures below 20 cm. (modified A. C. H.) does, however, represent an improvement. The lowest hip width measurement given in the American table is 20 cm., all cases with this measurement or a smaller one being pooled in a single group. Of the South Indian children nearly 50 per cent had hip widths below this figure. Similarly, the Indian data include a high proportion of cases with negative values of G, and it is preferable that negative values should, like positive values, be graded in relation to hip width.

The index could, however, be varied in many ways. The criterion table could be 'set' so that any given number of children should be selected, or so that a maximum number of children with signs should fall into the selected group. A possible development is the establishment of an index which would divide children in zones corresponding with a 'good', 'fair', and 'bad' state of nutrition. Presumably the characters which together correspond to 'selection' by the A. C. H. index disappear, not abruptly, but gradually on passing from severely malnourished children to children in a better state of nutrition. Children whose G values lie in the zone immediately above the A. C. H. line might be classed as 'fair' and those with G value above this zone as 'good'. Further research would be necessary to determine the best position of the line separating 'fair' from 'good' zones. Since a high percentage of children selected by the present index in South India are children with signs who could be assessed as malnourished by purely clinical means, a criterion which helped to assess the state of nutrition of children without signs would be very useful.

The application of the index to other age groups is also a problem requiring further study.

CONCLUSION.

The association between certain measurements of arm, chest, and hip, and the existence of deficiency disease elicited in these investigations opens up a new field for the study and detection of malnutrition. A deficient diet tends to cause certain changes in the structure and development of the body, and notably in the shape of the chest, which may be used to provide information about the state of nutrition of a child.

SUMMARY.

1. Four thousand six hundred and forty-six day school children in South India have been examined. Their A. C. H. measurements were taken and simultaneously the incidence of signs of deficiency disease—stomatitis, phrynodema, and Bitôt's spots—was recorded.

2. 15.9 per cent showed clinical signs and 23.8 per cent were 'selected'. The association between signs and 'selection' is shown by the fact that the index selected 76.7 per cent of those showing signs and only 13.9 per cent of those not showing signs.

3. Statistical analysis showed that the free and affected groups were not significantly differentiated in respect of age, arm girth, and hip width. On the other hand, average chest depth was greater in the affected children. It appears that, contrary to the views of Franzen and Palmer, an increased anterior-posterior diameter of the chest is the fundamental character on which the A. C. H. system is based.

4. Mean values of G (sum of arm measurements *minus* sum of chest measurements for a known hip width) were at all ages lower in the affected groups. Sixty per cent of affected children showed negative values of G as compared with 5 per cent of free children.

5. Height and weight means in free and affected children showed no significant differences.

6. The index is recommended as a useful supplementary method of assessing state of nutrition in South India. Certain suitable alterations are suggested and the possibility of further modification is considered.

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APPENDIX.

TABLE A.

Frequency distribution of age in free and affected cases.

Age group.	FIRST SURVEY.				SECOND SURVEY.	
	Boys.		Girls.		Boys.	
	Affected with clinical signs.	Free from signs.	Affected with clinical signs.	Free from signs.	Affected with clinical signs.	Free from signs.
5 to 6	17	..	6	..	2
6 to 7 ..	33	239	12	74	9	80
7 to 8 ..	61	430	13	69	22	149
8 to 9 ..	76	487	12	78	35	159
9 to 10 ..	93	457	12	70	32	140
10 to 11 ..	102	489	11	62	28	103
11 to 12 ..	69	360	2	41	20	81
12 to 13 ..	66	203	7	33	7	37
13 to 14 ..	13	26	3	5
14 to 15	4
15 to 16 ..	1	6
TOTAL ..	514	2,718	72	438	153	751

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TABLE B.

Frequency distribution : arm girth flexed (a) in free and affected groups.

Arm girth (a) flexed (cm.).	FIRST SURVEY.				SECOND SURVEY.	
	Boys.		Girls.		Boys.	
	Affected.	Free.	Affected.	Free.	Affected.	Free.
10-11 ..	1	1
11-12 ..	6	11	1	3	6	4
12-13 ..	40	160	1	9	18	67
13-14 ..	123	598	5	59	51	194
14-15 ..	142	775	22	117	49	237
15-16 ..	80	569	21	104	16	154
16-17 ..	62	331	9	77	9	56
17-18 ..	33	166	8	34	4	23
18-19 ..	18	62	2	19	..	10
19-20 ..	7	27	2	12	..	2
20-21 ..	1	12	1	2	..	3
21-22 ..	1	3	..	1	..	1
22-23	2	..	1
23-24	0
24-25	0
25-26	0
26-27	1
TOTAL ..	514	2,718	72	438	153	751

TABLE C.

Frequency distribution : arm girth extended (b) in free and affected groups.

		FIRST SURVEY.				SECOND SURVEY.	
Arm girth (b) relaxed (cm.).		Boys.		GIRLS.		Boys.	
		Affected.	Free.	Affected.	Free.	Affected.	Free.
10-11	..	1	1	..	1	3	1
11-12	..	28	97	1	7	7	27
12-13	..	94	524	4	43	42	161
13-14	..	159	817	19	117	61	268
14-15	..	98	643	25	118	24	190
15-16	..	72	374	10	74	12	66
16-17	..	36	172	9	38	3	24
17-18	..	19	61	1	28	1	9
18-19	..	4	19	3	6	..	2
19-20	..	2	7	..	4	..	3
20-21	..	1	2	..	2
21-22	0
22-23	0
23-24	0
24-25	1
TOTAL	..	514	2,718	72	438	153	751

TABLE D.

Frequency distribution : arm girth total (c) = (a) + (b) in free and affected groups.

Arm girth total (c)=(a)+(b) (cm.).	FIRST SURVEY.				SECOND SURVEY.	
	Boys.		Girls.		Boys.	
	Affected.	Free.	Affected.	Free.	Affected.	Free.
20-21 ..	1
21-22	1	..	1	3	1
22-23 ..	6	11	1	2	3	3
23-24 ..	20	66	..	2	4	18
24-25 ..	19	92	1	7	14	48
25-26 ..	66	313	2	28	25	89
26-27 ..	58	288	3	32	26	102
27-28 ..	90	473	8	54	35	145
28-29 ..	52	315	15	61	13	97
29-30 ..	56	378	12	65	12	113
30-31 ..	26	200	8	43	5	40
31-32 ..	40	217	6	36	7	45
32-33 ..	23	114	3	39	2	13
33-34 ..	19	109	5	23	2	19
34-35 ..	10	52	3	10	2	4
35-36 ..	11	36	2	14	..	6
36-37 ..	9	25	..	9	..	3
37-38 ..	4	6	1	5	..	0
38-39 ..	2	12	2	3	..	2
39-40	4	..	1	..	1
40-41 ..	1	3	..	1	..	2
41-42	1
42-43 ..	1	2	..	1
50-51	1
TOTAL ..	514	2,718	72	438	153	751

TABLE E.

Frequency distribution : Chest depth on expiration (d) in free and affected groups.

Chest depth (d) expiration (cm.).	FIRST SURVEY.				SECOND SURVEY.	
	Boys.		GIRLS.		Boys.	
	Affected.	Free.	Affected.	Free.	Affected.	Free.
9-10	2
10-11	60	..	8	..	8
11-12 ..	6	475	5	58	2	130
12-13 ..	51	942	12	136	21	348
13-14 ..	149	712	15	139	52	217
14-15 ..	190	381	29	73	60	45
15-16 ..	77	117	9	16	15	2
16-17 ..	33	27	2	7	3	1
17-18 ..	7	1	..	1
18-19
19-20 ..	1	1
TOTAL ..	514	2,718	72	438	153	751

TABLE F.

Frequency distribution : chest depth on inspiration (e) in free and affected groups.

Chest depth (e) inspiration (cm.).	FIRST SURVEY.				SECOND SURVEY.	
	Boys.		GIRLS.		Boys.	
	Affected.	Free.	Affected.	Free.	Affected.	Free.
10-11	11	..	4	..	1
11-12 ..	4	245	1	26	1	33
12-13 ..	25	822	7	113	7	269
13-14 ..	97	853	15	147	36	312
14-15 ..	197	493	26	112	65	116
15-16 ..	120	226	20	23	35	16
16-17 ..	48	59	2	11	7	4
17-18 ..	22	8	1	2	2	..
18-19
19-20 ..	1
20-21	1
TOTAL ..	514	2,718	72	438	153	751

TABLE G.

Frequency distribution: Chest depth total (f) = (d) + (e) in free and affected groups.

Chest depth total (f) = (d) + (e) (cm.).	FIRST SURVEY.				SECOND SURVEY.	
	Boys.		GIRLS.		Boys.	
	Affected.	Free.	Affected.	Free.	Affected.	Free.
19-20	1
20-21	9	..	4	..	1
21-22	45	0	4	..	5
22-23 ..	4	178	1	21	..	23
23-24 ..	2	286	4	37	2	91
24-25 ..	22	520	3	73	6	168
25-26 ..	26	414	9	56	13	173
26-27 ..	60	461	6	85	22	145
27-28 ..	83	256	8	55	29	90
28-29 ..	119	227	17	57	33	34
29-30 ..	72	151	12	21	28	16
30-31 ..	48	93	9	11	11	2
31-32 ..	34	39	1	5	5	2
32-33 ..	18	23	1	5	2	1
33-34 ..	14	11	1	3	2	..
34-35 ..	10	1	..	1
35-36 ..	1	2
36-37
37-38
38-39 ..	1
39-40	1
TOTAL ..	514	2,718	72	438	153	751

TABLE H.

Frequency distribution of G in free and affected groups.

$$G = a + b - (d + e).$$

G Range.	FIRST SERIES.				SECOND SERIES.	
	Boys.		Girls.		Boys.	
	Affected.	Free.	Affected.	Free.	Affected.	Free.
-7.9 to -7.0	1	..
-6.9 to -6.0	0	..
-5.9 to -5.0 ..	3	1	..
-4.9 to -4.0 ..	0	6	..
-3.9 to -3.0 ..	30	..	1	..	18	..
-2.9 to -2.0 ..	97	15	0	1	41	10
-1.9 to -1.0 ..	105	23	6	2	33	22
-0.9 to -0.1 ..	67	82	10	7	10	33
+0.0 to +0.9 ..	68	325	12	32	17	96
1.0 to 1.9 ..	51	531	11	57	9	159
2.0 to 2.9 ..	41	682	11	84	7	162
3.0 to 3.9 ..	34	468	5	97	6	139
4.0 to 4.9 ..	13	325	8	64	2	73
5.0 to 5.9 ..	0	156	4	45	2	28
6.0 to 6.9 ..	2	68	0	21	..	14
7.0 to 7.9 ..	1	24	3	17	..	8
8.0 to 8.9 ..	2	4	1	8	..	4
9.0 to 9.9	1	..	1	..	1
10.0 to 10.9	4	..	0	..	2
11.0 to 11.9	2	..	0
TOTAL ..	514	2,718	72	438	153	751

TABLE I.

Frequency distribution of hip width in free and affected groups.

HIP WIDTH (CM.).	FIRST SERIES.				SECOND SERIES.	
	Boys.		Girls.		Boys.	
Range.	Affected.	Free.	Affected.	Free.	Affected.	Free.
12.0-12.9	1
13.0-13.9	1
14.0-14.9	3
15.0-15.9	0
16.0-16.9 ..	1	17	..	10	..	1
17.0-17.9 ..	15	149	3	31	..	27
18.0-18.9 ..	44	305	7	63	13	102
19.0-19.9 ..	83	631	11	93	17	178
20.0-20.9 ..	131	717	14	81	42	239
21.0-21.9 ..	104	427	12	62	55	133
22.0-22.9 ..	68	238	11	40
23.0-23.9 ..	36	88	10	31	17	48
24.0-24.9 ..	22	32	0	14	7	18
25.0-25.9 ..	6	12	2	10
26.0-26.9 ..	1	3	2	3
27.0-27.9 ..	1	1
28.0-28.9 ..	2	2
29.0-29.9	0
30.0-30.9	1
TOTAL ..	514	2,718	72	438	153	751

TABLE J.

Frequency distribution : G¹.

G ¹ Range.	FIRST SERIES.		SECOND SERIES.	
	Boys.		Boys.	
	Affected.	Free.	Affected.	Free.
-4.0 to -3.0	1	..
-3.0 to -2.0 ..	3	..	1	..
-2.0 to -1.0 ..	27	3	26	..
-1.0 to -0.1 ..	114	24	44	11
0.0 to -1 ..	193	211	50	114
1 to 2 ..	90	1,029	18	279
2 to 3 ..	65	965	9	250
3 to 4 ..	13	400	4	71
4 to 5 ..	1	73	..	19
5 to 6 ..	2	8	..	5
6 to 7	5	..	2
7 to 8
TOTAL ..	514	2,718	153	751

TABLE K.

Frequency distribution of height and weight in free and affected children.

Range (in inches).	HEIGHT.				Range (in lbs.).	WEIGHT.			
	Boys.		GIRLS.			Boys.		GIRLS.	
	Free.	Affected.	Free.	Affected.		Free.	Affected.	Free.	Affected.
35-39.9	16	2	7	..	20-24.9	1	..
40-	115	30	50	5	25-	14	6	9	1
45-	230	57	76	11	30-	49	18	13	1
50-	235	59	36	4	35-	121	25	11	3
55-	54	16	6	..	40-	141	29	40	5
60-64.9	6	3	45-	140	37	36	5
					50-	101	9	15	2
					55-	74	19	8	2
					60-	44	15	7	0
					65-	20	5	4	1
					70-	4	0	1	..
					75-	1	2
					80-	3	1
					85-89.9	2	1
TOTAL ..	716	167	175	20	TOTAL ..	716	167	175	20

FURTHER STUDIES ON THE EFFECT IN RATS OF SUPPLEMENTING A SOUTH INDIAN DIET WITH CALCIUM AND PHOSPHORUS.

BY

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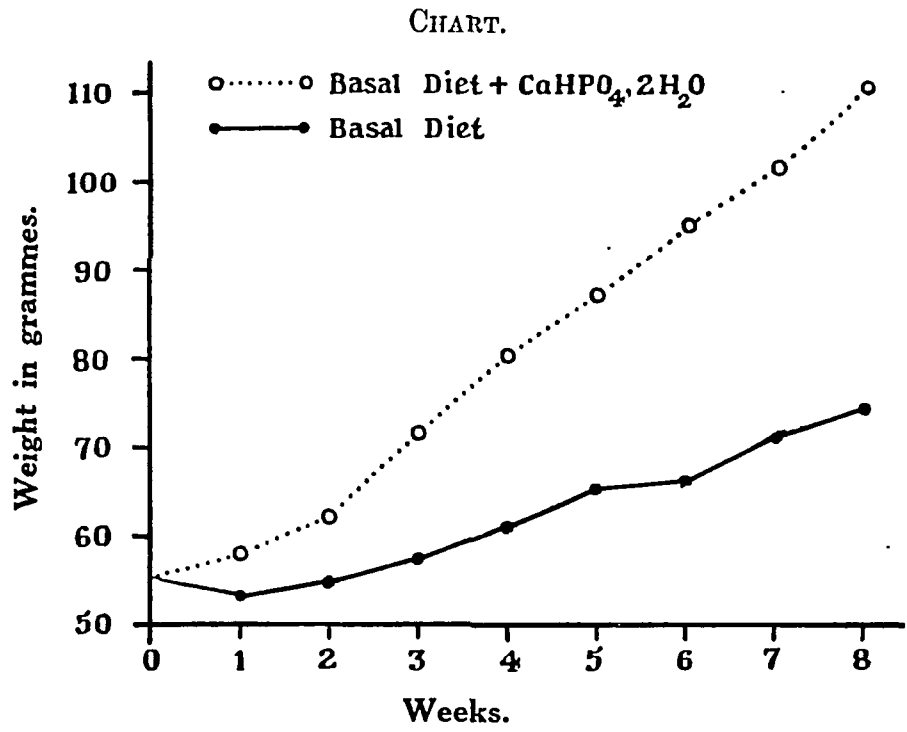
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It was shown by Aykroyd and Krishnan (1937) that the growth of rats fed on a diet resembling that consumed by the poorer classes in South India could be enhanced by the addition of calcium lactate. In a recent investigation (Pal and Singh, 1938), we confirmed and extended these observations, and studied calcium and phosphorus metabolism in rats fed on the same diet supplemented by calcium lactate and alkaline potassium phosphate separately or in combination. The present paper describes experiments in which a single salt, $\text{Ca HPO}_4 \cdot 2\text{H}_2\text{O}$, containing both calcium and phosphate, was given as a supplement to the poor South Indian diet based on rice. This salt is relatively cheap and might, if effective, be conveniently used as a supplement to the diets of school children, etc.

EXPERIMENTAL.

Two groups of animals, each consisting of 12 young rats, with equal numbers of males and females, of average weight 55 g., were used for the experiment. The basal diet was the 'poor South Indian diet' described in our previous paper (Pal and Singh, *loc. cit.*), which is largely composed of milled rice. One group was given this diet alone, the other group the same diet with the addition of 0.07 g. of alkaline calcium phosphate per rat per day as a supplement. We selected this salt as the most suitable one. According to Grulac (1922) neutral calcium phosphate $\text{Ca}_3(\text{PO}_4)_2$ is almost insoluble and probably not absorbed to a great degree.

The animals were kept separately in cages in a room exposed to the morning and evening sun. The experiment lasted for eight weeks during which weights were recorded every week. 0.5 c.c. of a saturated solution of alizarin red was injected into each animal twice weekly in order to determine the amount of calcium deposited in the growing bones of the animals at the end of the experiment. This procedure was suggested by the work of Salter and Aub (1931).



Showing the average rate of increase in weight of the two groups of experimental animals for eight weeks.

Table I shows the average weekly increase in weight of the two batches of experimental animals for eight weeks:—

TABLE I.

Increase in weight of groups of animals fed on a poor South Indian diet with (I) and without (II) alkaline calcium phosphate.
(Weight in grammes.)

Groups.	Initial weight.	1st week.	2nd week.	3rd week.	4th week.	5th week.	6th week.	7th week.	8th week.
I ..	55.20	53.00	54.70	57.25	61.00	65.50	66.50	71.50	74.70
II ..	55.20	58.20	62.30	71.70	80.50	87.60	95.20	101.75	110.40

Three animals from each group were removed to metabolism cages at the end of the second week and kept there for six weeks to study their metabolism. The dry weight of the food given was estimated every day and the rejected food, faeces, and urine of each animal were collected next morning. Intake and excretion of calcium phosphorus and nitrogen were estimated and their retention was calculated.

The estimation of calcium and phosphorus of food and faeces was done after ashing by the standard methods. That of phosphorus and calcium in urine was done by the combined method of Greenwald and Gross (1925). The nitrogen of the food and excreta was estimated by the Kjeldahl method.

At the end of the experimental period the length of the animals was measured (from the mouth to the end of the tail) under ether anaesthesia and then they were bled to death. Blood from the heart was collected from three animals of each group in one sample (as sufficient blood could not be obtained from one animal) for estimation of serum phosphorus by Brigg's (1922) modification of the Bell Doisy method, of serum calcium by the Kramer and Tisdall (1921) method and of protein by the macro-Kjeldahl method. The amount of non-protein nitrogen in blood, according to Frisch *et al.* (1929), is negligible and hence non-protein nitrogen was left out of account.

One femur of each animal was taken out for measuring the length and for investigating the extent of newly-formed bone as shown by staining with alizarin red. Table II gives the average body and femur length at the end of the experimental period :—

TABLE II.

*Average length of the animals from mouth to tail and
average length of the femur in the
two groups.*

Group	Final average length of the animal, cm.	Average length of the femur, cm.
I ..	25.5	2.23
II ..	30.0	2.62

The parathyroid glands embedded in the thyroids were also removed in order to observe any structural changes.

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The calcium, phosphorus and nitrogen contents of the food and excreta are recorded in Table III:—

TABLE III.

Intake, excretion, and retention of calcium (CaO), phosphorus (P₂O₅), and nitrogen (N₂) in the two groups of animals for six weeks.

Experi- mental week.	Group.	Total quantity of CaO in food.	Total quantity of CaO in excretion.	Total quantity of CaO in retention.	Total quantity of P ₂ O ₅ in food.	Total quantity of P ₂ O ₅ in excre- tion.	Total quantity of P ₂ O ₅ in reten- tion.	Total N ₂ in food.	Total N ₂ in ex- cretion.	Total N ₂ in reten- tion.
2nd week {	I	0.181	0.035	0.146	0.630	0.323	0.307	2.764	0.888	1.876
	II	0.603	0.109	0.494	1.214	0.581	0.633	3.230	1.187	2.043
3rd week {	I	0.161	0.047	0.114	0.494	0.361	0.133	2.186	1.247	0.939
	II	0.605	0.132	0.473	1.127	0.523	0.604	3.192	1.319	1.873
4th week {	I	0.207	0.023	0.184	0.529	0.293	0.236	2.292	1.064	1.128
	II	0.576	0.131	0.445	1.101	0.591	0.610	2.885	1.461	1.424
5th week {	I	0.185	0.036	0.149	0.551	0.358	0.193	2.285	1.574	0.711
	II	0.706	0.152	0.554	1.306	0.666	0.640	3.342	2.012	1.330
6th week {	I	0.166	0.023	0.143	0.562	0.302	0.260	2.570	1.298	1.272
	II	0.628	0.115	0.513	1.284	0.619	0.665	4.015	1.670	2.345
7th week {	I	0.174	0.058	0.116	0.590	0.429	0.161	2.672	1.393	1.279
	II	0.664	0.144	0.520	1.403	0.696	0.707	4.102	1.828	2.274

From the above table it can be noted that retention of calcium, phosphorus, and nitrogen was consistently greater in group I than in group II. The retention ratio of calcium and phosphorus as calculated from the CaO and P_2O_5 figures given in Table III shows a more or less constant figure between 1.2 and 1.42 for group II and a more variable one (0.9 to 1.6) for the other group :—

TABLE IV.

Calcium and phosphorus content per 100 c.c. of blood.

Group.	Calcium, mg. per cent.	Phosphorus, mg. per cent.
I	9.20	9.75
	8.80	11.60
	8.40	12.25
	8.80	10.90
AVERAGE ..	8.80	11.12
II	14.00	7.23
	14.48	7.21
	13.40	7.57
	13.60	7.38
AVERAGE ..	13.87	7.30

The blood protein showed no variation. The estimated quantity was 6.79 g. per cent for both groups.

The femurs, after being cut into two halves by a very fine saw and digested for four hours in a mixture of pancreatic trypsin and bile, showed staining by alizarin red at the epiphyseal ends of the bone. In the case of group II there was considerable staining of the diaphysis, while this staining was barely evident in the femurs of group I. During the process of sawing it was noticed that the bones of the former group were much harder than those of the other group, which were soft and could be easily cut through.

Histological examination of sections of the parathyroid glands of group II showed a normal structure, with compact principal cells, well-developed blood sinuses, and very scanty amount of fibrous tissue bands. In the glands of group I there were very big bundles of fibrous tissue with very few blood sinuses, suggesting hypo-function of the glands. The thyroids in this group showed some amount of colloid storage usually associated with the resting phase of the glands. The thyroids of the second group showed a normal structure.

DISCUSSION.

Increase in weight may not always be a completely satisfactory criterion of better growth and nutrition, and hence in this experiment we used other means to study developmental differences in the two groups. The group given alkaline calcium phosphate had the advantage not only as regards increase in weight but also as regards length of body, length of femur, and the amount of freshly deposited calcium at the growing ends of the femur.

The greater retention of calcium and phosphorus and also of protein by group II is further evidence of the superior nutrition of this group; addition of basic calcium phosphate seems to have improved the utilization of nitrogen. The more or less constant $\frac{\text{calcium}}{\text{phosphorus}}$ retention ratio for the second group is also indicative of better utilization of these elements by this group of animals.

Table IV shows the amounts of calcium and phosphorus in mg. per 100 c.c. of blood. The calcium content of the serum in the two groups of animals is in inverse ratio to that of phosphorus. The parathyroid glands revealed suggestive changes, those of group I showing a condition of hypo-function.

Peters and Eiserson (1929) found a direct relationship between serum calcium and protein but an inverse one between serum phosphorus and protein. They express the relation between the serum calcium, phosphorus, and protein by the following formula:—

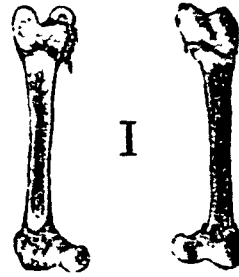
$$\text{Ca (in mg.)} = 7 - 0.566 \text{ protein (in g.)} - 0.255 \text{ P (in mg.)}.$$

Our figures do not, however, conform to this formula since the blood protein in both the groups was fairly constant and normal. According to Stearn and Knowlton (1931) this relationship holds good only when kidneys are damaged; in a series of experiments carried out by them on non-nephritic children, no relationship between the calcium, protein, and inorganic phosphorus content of serum was observed.

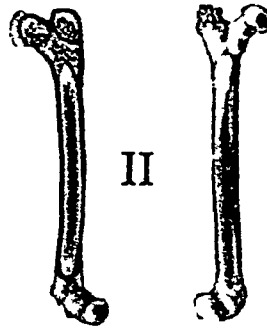
SUMMARY.

1. The nutritive value for rats of a diet based on rice resembling that consumed by the poorer classes in South India was improved by the addition of basic calcium phosphate. The improvement was evidenced by greater increase in weight, greater length of body and femur, and by more efficient calcification of bone.

2. The calcium content of the blood was greater in the group receiving the supplement than in the other group, the reverse being observed in the case of phosphorus.



I



II

Fig. 1.—I vital staining of the femurs by alizarin red; II showing considerable red staining of the epiphysis as well as diaphysis of the bones of group II animals having a supplement of alkaline calcium phosphate to their diet, in contrast to I, in which staining was barely evident in the bones of group I animals on basal diet alone.

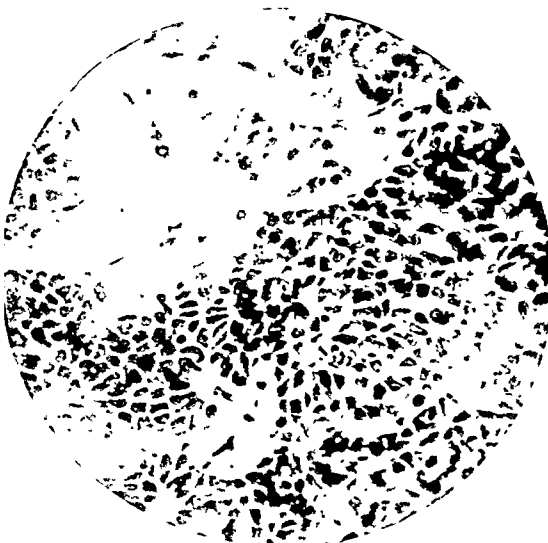


Fig. 2.—Parathyroid gland, group I, showing fibrosis and hypo-active condition of the gland.

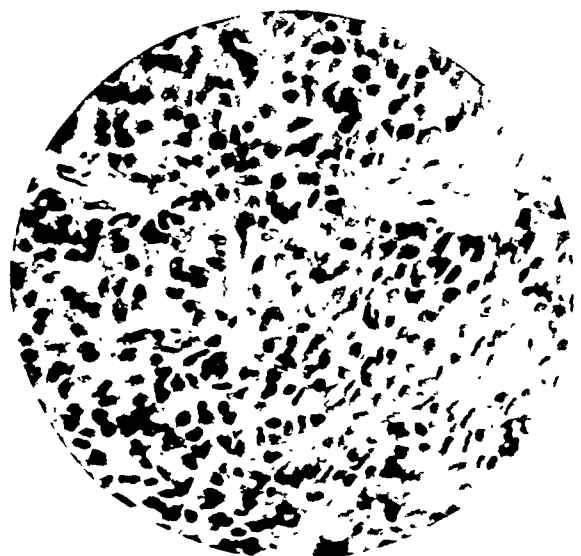


Fig. 3.—Parathyroid gland, group II, showing normal structure.

3. Changes suggestive of hypo-function were observed in the parathyroid glands of the animals on the basal diet alone. The parathyroids of those receiving the basic calcium phosphate supplement appeared to be normal.

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THE STUDY OF MYELIN DEGENERATION IN POLARIZED LIGHT.

BY

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SEVERAL histological methods have been devised for the study of myelin degeneration. Of these, Marchi's method, and staining with dyes of the Sudan type, are commonly used. These methods give positive pictures of degenerated myelin when applied during the earlier stages of the degenerative process, but their reliability, especially that of Marchi's method, has been questioned by several workers. A simple method involving the use of polarized light for the detection of myelin degeneration has recently been described by Setterfield and Sutton (1935), and Sutton, Setterfield and Krauss (1934). Baldi (1930) was the first to use polarized light for this purpose, but the credit of making practical use of the method must be given to the above-mentioned workers. Setterfield and Sutton (*loc. cit.*), applying the polarized light technique to the study of experimentally produced Wallerian degeneration in albino rats, concluded that the method is 'simple, speedy, and accurate'.

In the course of an investigation into the effects of avitaminosis A on the peripheral nervous system, it was felt that a simple and reliable method, which would bring out the changes with certainty in the earlier stages of the deficiency, was desirable. The present study was undertaken mainly to evaluate the polarized light technique. During the course of the investigation, the Marchi method was also critically studied using the same material.

METHOD.

The principle of the polarized light method of studying myelin degeneration depends on the physical and chemical properties of the myelin sheaths. The myelin sheath of a normal myelinated nerve fibre consists of a mixture of phospho-lipids (Mathews, 1930; Cramer and Lee, 1921), which are anisotropic (Cramer and Lee, *loc. cit.*); during the process of degeneration of the nerve fibre, the myelin is gradually changed from a mixture of phospho-lipids to a mixture of triglycerides (Hurst, 1925; Maximow and Bloom, 1934), which are isotropic (Cramer and Lee, *loc. cit.*).

When a longitudinal section of a myelinated nerve is observed in polarized light and between crossed Nicol prisms, degenerated myelin, if present, is seen as black areas (isotropic material), which in contrast to normal myelin (containing anisotropic material) do not show the property of birefringence—becoming

alternately light and dark in four positions at 90 degrees to one another when the object stage of the microscope is rotated through 360 degrees.

The principles underlying the use of this method for the detection of myelin degeneration have been discussed in detail by Setterfield and Sutton (*loc. cit.*) and Sutton, Setterfield and Krauss (*loc. cit.*).

A polarizing microscope is necessary for observing myelin degeneration by this technique. An ordinary microscope with a revolving stage can, however, be converted into a simple type of polarizing microscope for histological observations by fitting it with a polarizer and an analyser. The polarizer is placed in the diaphragm carrier of the illuminating apparatus below the stage, while the analyser is placed on top of the eyepiece. An interchangeable polarizer tube for stands D and F containing the analyser prism in a box-type mount has been introduced by Carl Zeiss, Jena. This tube is intended primarily for biological work and is neat and handy. The polarizer tube is inserted in place of the ordinary tube, and the polarizer is turned in relation to the analyser so that the field of view appears dark in the absence of an object.

MATERIAL FOR INVESTIGATION.

Experimental Wallerian degeneration in rabbits was studied by the above technique. Twelve healthy rabbits fed on a good mixed diet were used in the experiment. Under ether anaesthesia a small segment of the right sciatic nerve, at the junction of the proximal with the middle third, was removed and the wound in the thigh closed. The animals were killed by air embolism at various intervals (*vide* Table) after transection of the nerve. The distal part of the cut nerve

TABLE.

The progress of nerve degeneration after transection.

Serial number.	Rabbit number.	Interval after transection of the nerve.	LESIONS OF SCIATIC NERVES (MICROSCOPIC DIAGNOSIS).*	
			Marchi's method.	Polarizing light technique.
1	134	3 hours.	0	1
2	135	6 " "	1	1
3	136	9 " "	1	2
4	137	12 " "	2	2
5	118	24 " "	2	2
6	120	48 " "	2	3
7	121	3 days.	3	3
8	122	6 " "	3	4
9	125	9 " "	4	4
10	126	12 " "	4	4
11	127	15 " "	4	5
12	131	30 " "	5	5

* 0 = no degeneration. 1 = very slight degeneration.
 2 = slight degeneration. 3 = moderate degeneration.
 4 and 5 = marked degeneration.

was removed, trauma being avoided, soon after killing the animal and fixed in 10 per cent neutral formalin. As traumatic degeneration usually extends for a short distance in the cut ends of the nerve, pieces for examination were taken from the middle third of the distal segment. Twenty-four to forty-eight hours after fixation in formalin, longitudinal sections of the nerves at a thickness of 15μ to 20μ were cut on a Spencer's freezing microtome. The frozen sections were mounted in glycerin and examined in polarized light between crossed Nicol prisms.

OBSERVATIONS.

(See Plates II, III, and IV).

Sciatic nerves for control were taken from normal healthy rabbits and treated in the same manner. Figs. 1 and 2 (Plate II) give the appearances of longitudinal sections of the nerve when examined in polarized light between crossed Nicols. The myelin sheaths are doubly refractile and are about half the size of the axis cylinders. The nerve fibres are uniform in size and parallel to each other.

Sections of nerves examined *three and six hours* after transection showed an almost normal pattern; slight swelling was, however, present in some of the nerve fibres. Early degenerative lesions, shown by small areas of isotropic material, were noticeable in the myelin sheaths *nine hours* after sectioning of the nerve. The isotropic material in the myelin sheaths was more marked in the *twelve and twenty-four hours* specimens. The swelling of the nerve fibres was also more marked in these specimens, and the axis cylinders showed evidences of fragmentation in some fibres. The outlines of the fibres were prominent in the twenty-four hours specimen due to a thickening of the neurolemma, which is isotropic. There was an increase of isotropic material in the sections of nerves examined *two and three days* after transection; the normal myelin was much reduced in amount and isolated into a number of luminous islands separated by areas of isotropic material; swelling of the fibres and fragmentation were more marked than in the previous specimens. Examination of the nerves removed at the end of *six, nine, twelve, and fifteen days* after transection showed progressive increase in the number of fibres exhibiting degenerative changes, shown by fragmentation of the axis cylinders into separate segments of different lengths and increase of isotropic neutral fats. The fragments of axis cylinders became smaller and less in number with increase in the interval between transection of the nerve and removal of the distal segment for examination. After *30 days* only small, discrete masses of anisotropic material, representing myelin droplets, were present, the normal pattern of the nerve having completely disappeared; even the small segments of the axis cylinders were not visible in this specimen.

All the above specimens were also examined by the Marchi's method. The Table gives the results obtained by this method. By this technique, definite evidences of myelin degeneration were present *twelve hours* after transection of the nerve. Specimens of nerves removed after *three days* showed increasing amounts of degenerated myelin and an increasing number of degenerated nerve fibres. The results obtained by the Marchi method closely resemble those obtained by the polarized light technique.

DISCUSSION.

The progress of degenerative changes in the distal segments of the sciatic nerves following transection was clearly brought out by polarized light. Compared to the other methods commonly used for the study of myelin degeneration, this method is simple and rapid. It is useful in the study of early lesions occurring in myelinated nerves in avitaminosis. In a previous investigation (Radhakrishna Rao, 1936) it was applied to the study of lesions occurring in the trigeminal nerve in vitamin-A deficiency with satisfactory results. Recently, Lee and Sure (1937) used this technique in the study of degeneration in myelinated nerves in avitaminosis A and B.

Divergent views are held by different workers about the value of Marchi's method in the study of myelin degeneration. The results of this investigation suggest that it is a dependable method provided the technique is uniform throughout, and trauma to the nerves excised for examination and over-fixation in Müller's fluid are avoided.

SUMMARY AND CONCLUSIONS.

1. Experimentally produced Wallerian degeneration in the sciatic nerves of rabbits was observed in polarized light with a view to testing the reliability of the polarized light technique, described by Sutton, Setterfield and Krauss (*loc. cit.*), in the microscopic diagnosis of myelin degeneration.

2. The progress of the degenerative changes in the distal segments of the nerves following transection was clearly brought out when frozen sections of the nerves, cut longitudinally and mounted in glycerin, were observed in polarized light between crossed Nicol prisms.

3. The method is simple, rapid, and reliable.

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PLATE II.

E.—All sections of the rabbit nerves were photographed in polarized light between crossed Nicols at the point of greatest birefringence with 'Miflex' (Zeiss) camera attachment.



FIG. 1.

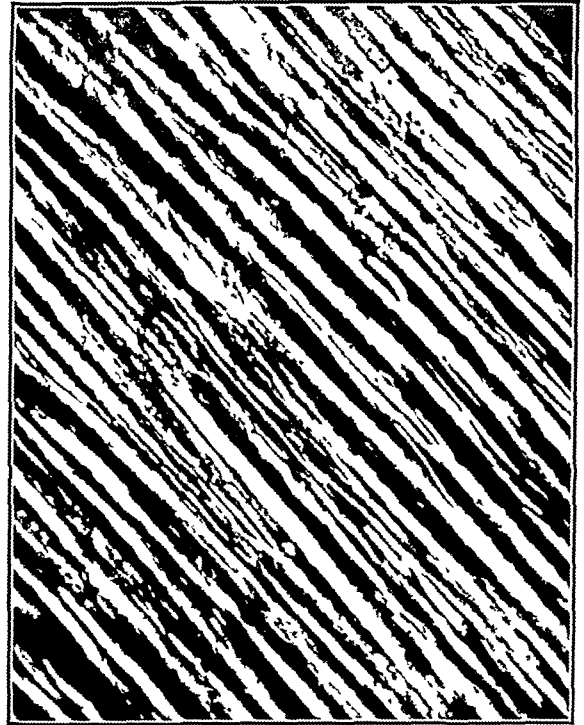


FIG. 2.

Figs. 1 and 2.—Photomicrographs of longitudinal sections of normal sciatic nerves. $\times 400$.



FIG. 3.

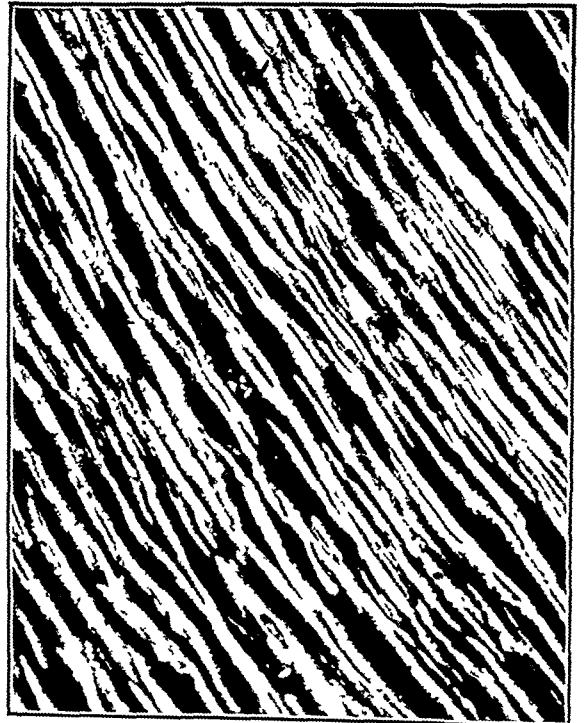


FIG. 4.

Figs. 3 and 4.—Photomicrographs of longitudinal sections of sciatic nerves 6 and 12 hours after transection. $\times 400$.



FIG. 5.



FIG. 6.



FIG. 7.



FIG. 8.

Figs. 5 to 8.—Photomicrographs of longitudinal sections of sciatic nerves 24 hours and 2, 3, and 6 days after transection.
× 400.

PLATE IV.



FIG. 9.



FIG. 10.



FIG. 11.

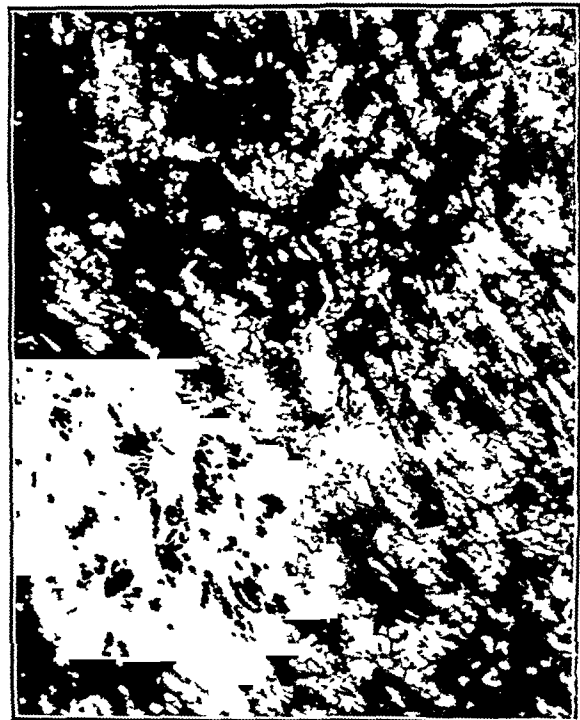


FIG. 12.

FIGS. 9 TO 12.—Photomicrographs of longitudinal sections of sciatic nerves 9, 12, 15, and 30 days after transection. $\times 400$

THE RELATIVE VALUE OF THE PROTEINS OF CERTAIN FOODSTUFFS IN NUTRITION.

Part V.

SUPPLEMENTARY VALUES OF THE PROTEINS OF *ELEUSINE* *CORACANA* (RAGI) AND OF CERTAIN PULSES AND SKIMMED MILK POWDER STUDIED BY THE NITROGEN BALANCE AND THE GROWTH METHOD.

BY

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PREVIOUS work (Swaminathan, 1937d) has shown that milk proteins from skimmed milk powder enhance appreciably the biological value of the proteins of rice and different pulses. Similar studies have been made with the proteins of ragi, a millet widely consumed in India and elsewhere. The results form the subject of this communication.

EXPERIMENTAL.

The moisture and 'crude protein' content of the test foodstuffs are given in Table I and the composition of the diets in Table II. The technique and the

TABLE I.

Moisture and 'crude protein' content of the test foodstuffs.

Name of foodstuff.	Botanical name.	Moisture.	'Crude protein' (N × 6.25), per cent.
Ragi	<i>Eleusine coracana</i>	13.05	7.40
Red gram	<i>Cajanus indicus</i>	12.54	23.44
Bengal gram	<i>Cicer arietinum</i>	12.86	22.26
Green gram	<i>Phaseolus radiatus</i>	12.19	24.82
Black gram	<i>Phaseolus mungo</i>	12.86	24.79
Lentil	<i>Lens esculenta</i>	12.46	25.13
Soya bean	<i>Glycine hispida</i>	8.50	40.04
Skimmed milk powder	4.10	38.55

TABLE IV.

The biological values of the mixed proteins of ragi and other foodstuffs obtained by the growth method during periods of 4 and 8 weeks, respectively, at an 8 per cent level of protein intake.

Sources of protein in the mixed diet.	BIOLOGICAL VALUE.	
	4 weeks.	8 weeks.
<i>Series I.</i>		
Ragi and skimmed milk powder	2.00	1.57
Ragi and red gram (dal arhar)	1.05	0.96
Ragi and Bengal gram	1.38	1.19
Ragi and green gram	1.50	1.23
Ragi and black gram	1.54	1.21
<i>Series II.</i>		
Ragi, red gram, and skimmed milk powder ..	1.48	1.38
Ragi, Bengal gram, and skimmed milk powder ..	1.98	1.78
Ragi, green gram, and skimmed milk powder ..	1.94	1.71
Ragi, black gram, and skimmed milk powder ..	1.88	1.50

DISCUSSION.

Ragi is an important millet in the Indian dietary, being consumed in large quantities by the poorer classes in the dry parts of India. Its protein content, though low (7.4 per cent), is comparable to that of rice; but its calcium content is very much greater than that of all the other common cereals and millets (Ranganathan *et al.*, 1937). Though the 'availability' of the calcium in ragi is relatively lower than that in other cereals and millets, bulk for bulk ragi may supply a greater amount of available calcium than rice and other cereals (Ranganathan, 1936). Aykroyd and Krishnan (1937) have observed that one of the chief deficiencies of the rice-eater's diet is calcium; ragi is, therefore, preferable to rice as a staple food for the poorer classes, provided its proteins are of good quality.

In a previous investigation (Swaminathan, 1937*a*) the proteins of ragi were found to be slightly superior to those of rice for the 'maintenance' of adult rats, while they proved to be inferior for the 'growth and maintenance' of young rats. Hence it might be expected that the biological value of the total proteins in a mixed diet of vegetable origin containing large quantities of ragi might be high for the 'maintenance' of nitrogenous equilibrium in adults, while the addition of

small quantities of milk proteins might enhance its value for the 'growth and maintenance' of young animals. These expectations have been to a large extent justified. The values of 71 to 84 obtained for the mixed proteins of ragi and different foodstuffs were further increased (84 to 94) on replacing 2 per cent of the pulse proteins by an equal amount of protein from skimmed milk powder. Similarly, values ranging from 0.96 to 1.57 obtained for the mixed proteins of ragi and pulses were increased (1.38 to 1.78) when 2 per cent of the pulse proteins were replaced by 2 per cent of milk proteins from skimmed milk powder.

The average weekly increase in body-weight observed in the groups of rats fed on the mixed diets containing ragi and rice as the principal cereals are given in Table V. In the majority of the cases, increase in body-weight was greater with the ragi diets. On the other hand, the biological values obtained by the growth method for the mixed proteins of the diets containing ragi are slightly lower in most cases than the corresponding values obtained with mixed diets containing rice. This is due to the greater food intake of rats fed on the ragi diets. Similar observations have been made by Mitchell and Hamilton (1929).

SUPPLEMENTARY RELATIONS OF RAGI AND RICE COMPARED.

Biological values for ragi and rice previously obtained by the nitrogen balance method were 89 and 80 respectively, those obtained by the growth method being 0.71 and 1.70 (Swaminathan, 1937a). The present investigation shows that ragi proteins, in conjunction with the proteins of pulses and milk, are somewhat superior to similar mixtures containing rice instead of ragi for 'maintenance' in adult rats, and of equal value for 'growth and maintenance' in young rats (Table VI). Evidently there is a satisfactory supplementary relation between the proteins of ragi, pulses, and milk.

TABLE V.

*Average weekly increase in body-weight during a period of 8 weeks
at an 8 per cent level of protein intake.*

Supplements added.	AVERAGE WEEKLY INCREASE IN BODY-WEIGHT, g.	
	Ragi + supplements.	Rice + supplements.
Skimmed milk powder	9.5	12.6
Red gram (dal arhar)	6.6	7.4
Bengal gram	8.3	6.8
Green gram	8.5	7.5
Black gram	8.3	5.9
Red gram + skimmed milk powder	9.5	8.2
Bengal gram + skimmed milk powder	12.5	9.9
Green gram + skimmed milk powder	11.8	10.9
Black gram + skimmed milk powder	10.0	8.3

TABLE VI.

Supplementary values of the proteins of ragi and rice in combination with those of different pulses and skimmed milk powder compared at an 8 per cent level of protein intake.

Supplements added.	BY THE NITROGEN BALANCE METHOD.		BY THE GROWTH METHOD.	
	Ragi + supplements.	Rice + supplements.	Ragi + supplements.	Rice + supplements.
Skimmed milk powder ..	94	80	1.57	1.86
Red gram (dal arhar) ..	84	76	0.96	1.26
Bengal gram	76	66	1.19	1.21
Green gram	81	56	1.23	1.29
Black gram	76	61	1.21	1.12
Red gram + skimmed milk powder	94	90	1.38	1.49
Bengal gram + skimmed milk powder.	88	79	1.78	1.89
Green gram + skimmed milk powder.	87	77	1.71	2.04
Black gram + skimmed milk powder.	89	80	1.50	1.39

The conclusion may be drawn that *large* quantities of animal proteins (e.g., milk proteins) are not required to raise the biological values of the proteins of a mixed diet based on ragi and other foods. Small amounts are of supplementary value.

SUMMARY.

Ragi proteins when supplemented by those of pulses and skimmed milk powder are very well suited for adult nutrition in rats, being slightly superior to those of rice given in similar circumstances. Ragi proteins by themselves were found to allow very little growth in young rats, but when supplemented by those of pulses and skimmed milk powder support good growth. In this respect mixtures of ragi and other proteins were found to be of almost equal value to corresponding mixtures containing rice.

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THE RELATIVE VALUE OF THE PROTEINS OF CERTAIN FOODSTUFFS IN NUTRITION.

Part VI.

GENERAL CONCLUSIONS.

BY

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THE nutritive and supplementary values of the proteins of different Indian foodstuffs have been the subject of five previous communications (Swaminathan, 1937a, b, c and d; 1938). In the present paper a short final summary of the results so far obtained is given together with certain unpublished results.

Two methods have been employed for assessing the relative value of the different proteins: (1) the nitrogen balance method for the 'maintenance' of nitrogenous equilibrium using adult rats and (2) the 'growth and maintenance' method using young rats. For details regarding the methods reference may be made to Parts I and II of this series. The biological values of the proteins of single foodstuffs, and the relative supplementary values of the proteins of mixed foodstuffs, are given in Tables I and II respectively.

All the results reported relate to the total nitrogenous constituents of the foodstuffs examined, referred to as their 'crude protein' content ($N \times 6.25$). Particular attention was given to proteins of vegetable origin, since animal proteins have been thoroughly investigated by previous workers (Boas-Fixsen, 1935) and shown to be in general of high biological value.

Cereals.—Cereal proteins, though inferior to animal proteins, possess high values. Six cereals were investigated. The proteins of rice were found to be of the highest biological value for the growth of young rats, while the proteins of ragi proved to be the best for 'maintenance' in adult rats.

Pulses.—Pulses in general contain a high percentage of protein and so form an important source of proteins in Indian diets; these proteins are, however, of relatively low biological value. Nine common pulses were investigated. The proteins of red gram were found to be the best for 'maintenance' of nitrogenous equilibrium in adult rats, while the proteins of black gram supported good growth in young rats.

TABLE I.

Biological value and digestibility co-efficients of the proteins of 27 foodstuffs.

Name of foodstuff.	Botanical name.	BY THE NITROGEN BALANCE METHOD.			BY THE GROWTH METHOD.	
		'Crude protein' content, per cent.	Biological value, per cent.	Digestibility co-efficient, per cent.	'Available protein', per cent.	Biological value, per cent.
<i>Cereals (at 5 per cent level of protein intake).—</i>						
Cambu ..	<i>Pennisetum typhoideum</i>	10.48	83	89	7.74	1.15
Cholam ..	<i>Sorghum vulgare</i>	10.27	83	91	7.76	0.78
Italian millet ..	<i>Setaria Italica</i>	10.02	77	91	7.02	..
Ragi ..	<i>Eleusine coracana</i>	7.12	89	80	5.07	0.71
Rice, raw milled ..	<i>Oryza sativa</i>	6.88	80	97	5.34	1.70
Wheat, whole ..	<i>Triticum vulgare</i>	12.62	66	93	7.75	1.31
<i>Pulses (at 10 per cent level of protein intake).—</i>						
Bengal gram ..	<i>Cicer arietinum</i>	22.10	62	86	11.78	0.70
Black gram ..	<i>Phaseolus mungo</i>	23.50	62	78	11.36	1.00
Cow gram ..	<i>Vigna catiang</i>	24.12	45	78	8.47	..
Field bean, dry ..	<i>Dolichos lablab</i>	24.63	41	76	7.67	..
Green gram ..	<i>Phaseolus radiatus</i>	23.80	51	86	10.44	0.94
Horse gram ..	<i>Dolichos biflorus</i>	22.12	66	73	10.65	..

Lentil	25.70	41	89	9.27	0.47
Red gram	22.65	72	75	12.23	..
Soya bean	40.00	54	76	16.42	0.78
<i>Nuts and oilseeds (at 10 per cent level of protein intake).—</i>							
Cashew nut	21.19	72	90	13.73	..
Coco-nut	4.47	77	94	3.24	1.03
Gingelly seeds	18.33	67	85	10.44	1.03
Ground-nut	26.72	57	90	13.46	..
<i>Vegetables (at 5 per cent level of protein intake).—</i>							
Amaranth leaves	4.90	72	78	2.75	..
Brinjal	1.31	71	75	0.75	..
Cluster beans	3.67	51	73	1.37	..
Drumstick leaves	6.65	41	77	2.72	..
Ipomea leaves	2.90	67	85	1.65	..
Lady's finger	2.20	82	70	1.26	..
Sebania leaves	8.40	64	85	4.55	..
<i>Milk.—</i>							
Skimmed milk powder (at 5 per cent level of protein intake).	38.04	89	90	30.47	2.03
Skimmed milk powder (at 10 per cent level of protein intake).	38.04	83	90	28.42	1.45

TABLE II.

Supplementary values of the proteins of ragi and rice in combination with those of different pulses and skimmed milk powder at an 8 per cent level of protein intake.

Supplements added.	BY THE NITROGEN BALANCE METHOD.			BY THE GROWTH METHOD—8 WEEKS.	
	DIGESTIBILITY CO-EFFICIENT.		BIOLOGICAL VALUE.		
	Ragi — supplements.	Rice — supplements.	Ragi — supplements.	Rice — supplements.	
Series I					
Skimmed milk powder ..	93	89	94	1.57	1.86
Red gram (dal arhar) ..	83	81	84	0.96	1.26
Bengal gram ..	84	85	76	1.19	1.21
Green gram ..	86	90	81	1.23	1.29
Black gram ..	81	82	76	1.21	1.12
Lentil ..	91	..	71
Soya bean ..	82	84	71	..	1.13
Series II					
Red gram + skimmed milk powder	92	87	94	1.38	1.49
Bengal gram + ..	87	96	83	1.78	1.89
Green gram + ..	91	95	87	1.71	2.04
Black gram + ..	82	92	89	1.50	1.39
Lentil + ..	90	..	84
Soya bean + ..	83	93	87	..	1.33

Nuts and oilseeds.—Oilseeds and nuts are rich in protein, comparable in this respect with pulses. The proteins of nuts and oilseeds were found to be of fairly high biological value for 'maintenance' in adult rats.

Vegetables.—Though vegetables do not contribute largely to the total proteins of the diet, they are, in proportion to their calorie yield, surprisingly rich in protein. Of the seven vegetables investigated five were found to contain proteins of fairly high biological value.

SUPPLEMENTARY VALUES.

Recent investigations have shown (Rose, 1938) that out of the 22 known amino-acids, 10 are essential for growth and maintenance in the sense that they must be supplied by the food protein. No foreign protein contains all the essential amino-acids in the required proportions. Since the biological value of a protein depends largely upon its amino-acid make-up, the proteins of two or more food-stuffs may be able to make good one another's deficiencies, so that a mixture containing equal quantities of two proteins may have a biological value which is greater than the arithmetical mean of their individual biological value. The total protein in an ordinary diet consists of a mixture of proteins derived from foodstuffs of both vegetable and animal origin.

The addition of small quantities of milk protein enhances the biological value of a mixture of proteins derived from cereals and pulses. Ragi proteins by themselves were found to produce very little growth in young rats, being inferior in this respect to rice (Table I); but when ragi and rice proteins are supplemented by the proteins of pulses and milk, values obtained for the protein mixtures are almost the same.

BIOLOGICAL VALUE OF PROTEINS AND 'WELL-BALANCED' DIETS.

The unequal nutritive values of proteins are of importance in planning 'well-balanced' diets. For practical purposes, nutrition workers are accustomed to divide proteins broadly into two classes, the first including proteins of animal origin and the latter all the others. The minimum amount of animal protein required for optimum nutrition is still a matter of uncertainty. The results obtained in the present series of investigations suggest that *at least* 25 per cent of the total proteins in a diet should be of animal origin.

SUMMARY AND CONCLUSION.

1. The biological values of the proteins of 27 common Indian foods, including 6 cereals, 9 pulses, 4 nuts and oilseeds, 7 vegetables, and skimmed milk powder, and the supplementary relations between the proteins of rice and ragi and other foods, have been investigated.

2. Though the proteins of a mixed diet of vegetable origin are of fairly high biological value for 'maintenance' of nitrogenous equilibrium in adult rats, small

amounts of animal proteins (e.g., milk proteins) are probably necessary for satisfactory growth in young rats.

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VARIATIONS IN THE IRON CONTENT OF FOODSTUFFS AND THE PROBLEM OF IRON REQUIREMENTS.

BY

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THE importance of iron in nutrition is well known. In practical nutrition work the elements which usually receive the greatest attention are calcium, phosphorus, and iron, these being elements which are likely to be insufficiently present in human diets. In assessing the general adequacy of diets, it is usual to work out their mineral content from tables of food analyses, and compare intake so calculated with standards such as those suggested by Sherman (1937). In diet surveys in India, intake of calcium, phosphorus, and iron has been calculated using the data of Ranganathan *et al.* (1937), which cover 200 foodstuffs. The figures given by these workers have been incorporated in Health Bulletin No. 23 (1937), 'The Nutritive Value of Indian Foods and the Planning of Satisfactory Diets', which is now being used in nutrition work throughout India.

In the case of calcium and perhaps of phosphorus, the procedure outlined above is a reasonable one. But with iron the position is more complicated for a number of reasons. The most important of these is local variation in the iron content of foodstuffs, presumably related to differences in soil and climatic conditions. Another is the possibility of contamination of foodstuffs with iron during the process of preparation and cooking which may generally increase iron content. A third is the uncertainty regarding iron requirements. Finally, there is the problem of the 'availability' of iron contained in various foodstuffs. These questions will be separately discussed.

VARIATION IN THE IRON CONTENT OF FOODSTUFFS.

Tables showing the iron content of foodstuffs commonly consumed in Europe and America are provided by Peterson and Elvehjem (1928), Wallgren (1932), Shackleton and McCance (1936), Mottram and Radloff (1937), and Sherman (*loc. cit.*); for foodstuffs common in the East there are the figures of Rosedale (1935) (Malaya), Ranganathan *et al.* (*loc. cit.*) (India), Read *et al.* (1937) (China), and Nicholls (1937) (Ceylon). The accurate determination of iron in biological materials

TABLE I.

The iron content of foodstuffs as reported by different workers.

Foodstuff.	Health Bulletin.	Ranganathan.	Nicholls.	Rosedale.	Read <i>et al.</i>	Sherman.	Shackleton and McCance.	Mottram and Radloff.	Peterson and Elvehjem.	Wallgren.
Barley ..	3.70	4.10	..	3.58	3.58	..
Maize, dry ..	2.10	2.08	1.50	2.90	2.02
Oatmeal ..	3.81	4.33	3.80	4.15	3.80	3.80	5.10
Rice, raw, milled ..	1.02	2.22	..	1.02	1.00 to 8.50	0.90	0.45	1.05	1.05	0.67
Wheat, whole ..	5.34	3.97	..	2.00	..	5.00	3.72	1.45
Refined wheat flour ..	1.00	1.53	1.00	1.00	0.74
Green gram (<i>Phaseolus radiatus</i>).	8.40	10.00	..	14.00	9.70
Lentil ..	1.98	1.98	7.63	7.62
Cabbage ..	0.76	0.73	..	0.66	0.60 to 0.70	0.43	0.98	..	0.34	0.19
Celery ..	6.25	2.7 to 5.20	0.62	0.14	0.61	0.77	..
Lettuce ..	2.39	2.39	1.20 to 2.70	0.70	0.80	0.73	1.97	0.45 to 0.66
Parsley ..	17.86	10.00	..	19.21	4.85
Spinach ..	4.95	5.00	..	4.32	19.50	2.55	2.96	..	6.60	1.20 to 4.78
Beet-root ..	0.98	0.98	2.22	0.85	0.50	1.83
Carrot ..	1.52	2.83	..	0.75	1.90	0.64	0.56	0.56	1.07	0.20

Parsnip ..	0.10	0.54	..	0.50	0.77	0.78	0.57	1.07	0.47
Potato ..	0.68	0.76	..	1.30	0.91	0.60	0.75	0.85	0.23 to 0.33
Radish ..	0.1 to 0.47	..	3.80	0.40	0.83	1.68	1.88	1.36	..
Brinjal (<i>Solanum melon-</i> <i>gena</i>). ..	1.31	0.44	..	1.40	0.50	0.32	..	0.61	..
Cauliflower ..	1.25	1.38	..	1.20	0.94	0.91	..	1.43	0.52
Cucumber ..	1.45	..	1.00	1.00	0.33	0.25	0.30	0.35	..
French beans ..	1.67	1.67	..	1.80	..	0.57	0.59	..	0.98
Leeks ..	2.30	8.90	0.65	0.77
Peas, English ..	1.47	1.90	..	0.10	2.07	1.77	1.88	1.77	1.49
Pumpkin ..	0.67	0.50	0.93	..	0.39	1.10	..
Rhubarb stalks ..	2.20	0.56	0.38	0.40	0.86	0.22
Tomato, green ..	2.37	..	1.50 : 2.30	0.90	0.44	0.37	..	0.60	..
Turnip ..	0.42	1.80	0.52	0.33	0.35	0.70	..
Ground-nut ..	1.56	1.63	..	2.00	2.00	1.19	2.04	2.31	..
Walnut ..	4.76	3.50	2.10	1.83	2.35	5.98 (Black) 2.14 (English)	2.64
Apple ..	1.68	0.73	..	1.00	0.36	0.23	0.29	0.43 (Average)	0.14
Banana ..	0.42	0.80	0.61	0.47	0.41	1.76	0.19
Grapes, blue variety ..	0.40	1.40 to 3.00	0.73	0.27	0.34	..	0.37
Orange ..	0.08	0.53	..	0.10 to 2.00	0.52	..	0.33	..	0.10 to 0.26
Peaches ..	1.70	0.80	0.33	0.39	0.33	0.36	..
Pineapple ..	0.92	1.50	0.37	0.22	0.42	0.32	..

TABLE I--concl'd.

Foodstuff.	Health Bulletin.	Ranganathan.	Nicholls.	Rosedale.	Read et al.	Sherman.	Shackleton and McCanee.	Mottram and Radloff.	Peterson and Elvehjem.	Wallgren.
Plums ..	0.55	1.80	0.56	0.35	0.36	0.77	0.18
Pomegranate ..	0.27	1.60	0.78	0.20	..	1.17	..
Pomelo ..	0.04	0.46	..	0.50	0.90
Strawberry ..	1.75	1.10	0.68	0.71	0.71	0.66	0.58
Tomato, ripe ..	0.08	0.44	0.37	0.43	..	0.27 to 0.31
Beef, muscle ..	0.77	2.10	..	2.60	2.10	3.00	3.54	9.80 (corned).	..	1.85; 1.49; 2.35
Mutton, muscle ..	2.52	3.16	..	3.00	2.00	2.76	0.014 to 0.032
Milk, cow's ..	0.24	0.24	0.10	0.24	0.24	0.24	1.38	(Average 0.024) 0.65
Raspberries	0.88	1.11	1.21	0.99	..
Watercress	2.97	2.08	1.62	7.21	..
String beans	0.50	1.60	1.10	9.52	..
Apricots	1.60; 3.30	0.60	4.08; 0.37	0.37	7.26 (dry)	..
Cherries, red	0.40	0.48	0.38	0.46	0.37
Cocoa	2.70	14.20	3.13	3.13	10.20; 74.35
Currants, dried	4.00	1.07	1.27	4.74	0.44 (Fresh)
Gooseberries	0.50	0.58	0.32	0.48	0.32

was a serious problem until a few years ago, but with the advent of the ferric thiocyanate-amyl-alcohol and other equally refined methods, technical difficulties have been almost overcome. Differences in the figures for the iron content of the same foodstuffs given by various workers cannot be ascribed to the methods of analysis.

Examples of variation in the iron content of different samples of the same foodstuff are reported in the literature. Peterson and Elvehjem (*loc. cit.*) found that certain samples of a given foodstuff may be three times as rich in iron as other samples. Thus, 20 samples of cabbage analysed by them showed an iron content varying from 0.17 mg. to 0.59 mg. per cent. Similar marked variations have been reported by Coons and Coons (1935), Toscani and Rezinikoff (1934), and Davidson and LeClerc (1936). Such differences may presumably often be related to the iron content of the soil in which the foodstuffs analysed were grown.

The selection of samples may be of importance. Peterson and Elvehjem (*loc. cit.*) have observed a direct relationship between iron content and the presence of chlorophyll. For this reason tender growing shoots tend to give higher iron values than those given by maturer parts of the same plant.

Table I demonstrates the range of variation in values given by different workers. In constructing the table no attempt was made to select only foodstuffs for which a widely divergent iron content has been recorded. The selection was a fairly random one, the only criterion for inclusion being the reporting of a figure by four or more workers.

It will be seen that there is fair correspondence in the values for the iron content of cereals, pulses and meat given by the different investigators. There are, however, wide divergencies in the values for other groups of foodstuffs, notably leafy vegetables. Thus, the figure for celery ranges from 0.14 mg. to 6.25 mg. per cent; for lettuce from 0.45 mg. to 2.70 mg.; for parsley from 4.85 mg. to 19.21 mg.; and for potato from 0.23 mg. to 2.00 mg. per cent. In general, the figures given by workers in the East are higher than those given by European and American workers, and this appears in general to apply to foodstuffs other than those listed in Table I.

Table II gives a series of values for cow's milk collected from the literature:—

TABLE II.

Iron content of cow's milk as reported by various workers.

Author.				Date.	Iron content, mg. per 100 c.c.
Sherman	(quoted by Stearns and Stinger, 1937)	..		1907	0.24
Edelstein-Csonka	do.	do.	..	1911	0.05
Peterson and Elvehjem	do.	do.	..	1928	0.24

TABLE II—concl'd.

Author.				Date.	Iron content, mg. per 100 c.c.
Telfer	(quoted by Stearns and Stinger, 1937) ..			1930	0.07 to 0.11 ; average 0.08
Cunningham	do.	do.	..	1931	0.065
Davies	do.	do.	..	1931	0.15 to 0.38
Stugart	do.	do.	..	1931	0.044 to 0.073
Reis and Chakmakjian	do	do.	..	1932	0.14 to 0.15
Wallgren	do.	do.	..	1932	0.014 to 0.032 ; average 0.024
Rowett Research Institute	do.	do.	0.07 to 0.11
Department of Pædiatrics, Coll. of Med. State Univ., Iowa (quoted by Stearns and Stinger, <i>loc. cit.</i>) ..				1935	0.038 to 0.072
Svedenins	(quoted by Wallgren, <i>loc. cit.</i>) ..			1929	0.163 to 0.09
Lesne-Clement Zizine	do.		..	1930	0.09 to 0.10

It is clear that widely different figures for the iron content of human diets would be obtained by the use of the data provided by the various investigators, and this would be particularly so if maximum and minimum figures were used. This point is illustrated by the following: A diet containing eight ingredients, based on cereals, a pulse, vegetables, fruit, and butter, and resembling an ordinary human diet in its general composition, was devised. Most of the foodstuffs included are commonly consumed in both Western and Eastern countries. The energy value of the diet is about 2,500 calories, roughly equivalent to adult requirements. The iron content of this diet, as calculated on the basis of the figures supplied by the various workers, is given in Table III. In a few instances values for certain of the foodstuffs included in the diet were lacking in the lists given by the various workers; in such cases the figures of Ranganathan *et al.* (*loc. cit.*) or of Sherman (*loc. cit.*) have been used. The values in question are marked (R) and (S), respectively.

The iron content of the diet is found to vary from 10.703 mg. to 51.034 mg. If a standard requirement of 15 mg. were adopted, the diet would appear to be deficient in iron if its iron content were calculated from Wallgren's figures. According to the figures of Shackleton and McCance, Sherman, and Mottram and Radloff, it would be in the borderline of sufficiency, while according to the data provided by the other workers it appears to contain an abundance of iron. These discrepancies would be even more exaggerated had the diet included more green leafy vegetables.

TABLE III.

The iron content of a diet as calculated from figures furnished by different investigators.

Ingredients of diet, oz.	Health Bulletin.	Sherman.	Rosedale.	READ <i>et al.</i>		Shackleton and McCance.	Mottram and Radloff.	Peterson and Elvehjem.	WALLOREN.	
				Min.	Max.				Min.	Max.
Rice 10	2-887	2-556	2-887	2-840	24-140	1-278	2-982	2-982	1-903	1-903
Wheat 5	7-583	7-100	2-840	2-840	2-840	7-100 (S)	7-100 (S)	5-282	2-059	2-059
Milk 8	0-545	0-545	0-545 (S)	0-227	0-227	0-545	0-545	0-545	0-545 (S)	0-545 (S)
Red gram (<i>Cajanus indicus</i>) 3	7-498	4-737 (R)	4-737 (R)	4-430	4-430	4-737 (R)	4-737 (R)	4-737 (R)	4-737 (R)	4-737 (R)
Spinach 3	4-217	2-173	3-681	16-614	16-614	2-522	2-173 (S)	5-623	1-039	4-063
Potatoes 6	1-139	1-551	3-403	2-215	2-215	1-022	1-278	1-448	0-392	0-562
Orange 1	0-023	0-148	1-079	0-114	0-568	0-148 (S)	0-094	0-148 (S)	0-028	0-074
Butter 1-5	0	0	0	0	0	0	0	0	0	0
TOTAL	23-912	18-810	19-177	29-280	51-034	17-352	18-909	20-765	10-703	13-943

R = Ranganathan ; S = Sankaran.

In practical dietetics, workers naturally tend to use tables of food values prepared in their own country, and when this is done, differences between the calculated and actual iron content of diets may be relatively small, since foods analysed and foods consumed will tend to be generally similar in origin. It is, however, impossible to gauge the extent of such differences. In a huge country like India, local variation in the iron content of the same foodstuff may be very considerable. Most of the foodstuffs analysed in the Laboratories were grown in the neighbouring plains of the Coimbatore district. The iron figures obtained have been used in diet surveys two or three thousand miles away in North and North-East India. Table III certainly suggests that such a procedure is unlikely to produce valid results.

It is noteworthy that similar variations are not found in the figures for the calcium and phosphorus content of foodstuffs reported by different workers.

THE CONTAMINATION OF FOODSTUFFS WITH IRON.

In the preparation of food samples for iron analysis, the laboratory worker uses a stainless steel knife or some non-ferrous implement to prevent iron contamination. In households ordinary knives are used, and food may be put in iron vessels. Experience in this Laboratory has shown that the accretion of iron which may result from iron contamination during storage and preparation may be very considerable. Wallgren (*loc. cit.*) reports that cow's milk stored in glass vessels contained 0.0226 mg. per cent of iron, while the same milk stored for a similar period in tin vessels was found to contain 1.225 mg. per cent. Langstein, Edelstein and Csonka (quoted by Wallgren, *loc. cit.*) also report similar findings; they reported that cow's milk kept in glass vessels contained 0.03 mg. to 0.07 mg. and the same milk kept in tin vessels 0.064 mg. to 0.122 mg. per cent.

REQUIREMENTS OF 'TOTAL' IRON.

The amount of iron required to maintain a positive balance is not known with any certainty. Sherman's standard of 15 mg. per adult per day has been widely used in public health nutrition work. In the latest (1937) edition of 'The Chemistry of Food and Nutrition' he has reduced the standard requirement to 12 mg. The range of values given by different workers, representing the daily intake of iron needed to preserve a positive balance in individuals, is very high. Thus, Lintzel (1931) has observed a positive balance with an intake of 1 mg. and less; Farrar and Goldhamer (1935) with an intake of about 5 mg., while Ohlson and Daum (1935) recorded a negative balance with an intake of 13.78 mg.

Orten, Smith and Mendel (1936) observed that the utilization of iron was improved by an increased intake of calcium. Iron requirements are of course increased in anæmia and in conditions in which destruction and loss of red blood corpuscles occur, such as hookworm and malaria. In India and other Eastern and tropical countries there may be a high incidence of such conditions among populations whose diet is the object of study.

'AVAILABLE' IRON.

The problem of iron intake and requirements has been further complicated by the introduction of the concept of 'available' iron, first propounded by Hill

(1930) and later extended by Elvehjem and his co-workers (1933, 1934, and 1936). 'Available' iron is taken by these workers to be equivalent to the simpler, inorganic or ionizable iron moiety of foodstuffs which reacts with dipyridine and which alone is responsible for hæmoglobin regeneration, as opposed to the more complex iron fraction in foodstuffs which has no physiological significance. Considerable work has lately been done on 'available' iron and the 'available' iron content of foodstuffs has been reported by several workers. Here there is the same divergence between the results of the different investigators as in the case of total iron. The 'available' iron content of a foodstuff is usually represented as a percentage of its total iron, and it is therefore logical to expect differences, in view of similar differences in the total iron content. But even in the absolute amounts of 'available' iron present in foodstuffs, wide variations exist.

Table IV gives the figures for 'available' iron, expressed as per cent of total iron, for a few foodstuffs, randomly selected :—

TABLE IV.

'Available' iron in some common foodstuffs as reported by various workers.

(Per cent of total iron.)

Foodstuff.	Köhler, Elvehjem and Hart (1936).	Shackleton and McCance (<i>loc. cit.</i>).	Ranganathan (1938).
Wheat ..	47	..	43
Oats ..	57	..	43
Peas, fresh ..	72	74	78
Soya bean ..	80	..	29
Parsley ..	23	50	18
Lettuce ..	25	63	42
Spinach ..	19	68	19
Apricots ..	50	98	..
Almonds ..	88	99	43
Bananas ..	61	100	47
Beef ..	50	11	35
Cabbage	62	38
Potato	95	45
Rice	85	33
Yeast ..	47	..	43

Hahn and Whipple (1938) have recently adduced strong evidence showing the untenability of the 'available' iron concept of Hill and his followers. Ionizable iron salts such as ferric or ferrous salts react quantitatively with dipyridine and hence are 100 per cent 'available'. Yet when given *by mouth* to anaemic animals in optimum amounts, these salts were found to be only 35 per cent 'available', whereas the same quantities given *intravenously* are 100 per cent available for haemoglobin formation. The same workers have shown that availability decreases with increasing dosage. Apricots are more efficient in haemoglobin regeneration than their iron content would appear to warrant, while fresh liver gives rise to twice as much haemoglobin as does its ash. On the basis of observations of this nature, Hahn and Whipple have introduced the term 'physiological availability' of iron, in contradistinction to 'availability' as determined by the dipyridine method, the application of which has led to numerous fallacies.

More work is needed to clarify the problem of 'available' iron. The point which it is desired to make here is that the existence of the problem throws further doubt on the value of computations of total iron intake and requirements from tables of food analyses.

CONCLUSION.

In view of these facts, it is felt that calculations of the iron content of dietaries from the figures given in such publications as Health Bulletin No. 23 are of very dubious value. The Bulletin suggests that 'a well-balanced diet' for a growing child or an adult should contain 20 mg. of iron according to the tables. This figure gives a 'margin of safety'. There is probably no serious danger in adopting the Health Bulletin standard. Nevertheless the facts put forward in this paper suggest that at present there is little point in carefully calculating the iron content of diets from data obtained in diet surveys or that of diets drawn up for institutions, etc. If a diet contains protein, calcium, phosphorus, and various vitamins in reasonably adequate amounts, its iron content will probably be adequate; if it is ill-balanced and generally deficient, e.g., based almost exclusively on milled rice, it is likely to be deficient in iron as well as in other food factors. At present no greater degree of accuracy appears possible.

SUMMARY.

1. The practice of computing the iron content of human dietaries from tables of food analyses has been critically considered.
2. The variations in the iron content of foodstuffs reported by different workers are described. The effect of such variations in the computation of the iron content of human dietaries is demonstrated.
3. In laboratory determinations of the iron content of foodstuffs special precautions are taken to avoid iron contamination. Calculations of the iron content of diets from figures in food value tables do not take into account the accretion of iron through contamination during the storage and preparation of foods, which may be very considerable.

4. The problem is further complicated by conflicting data regarding total iron requirements, and by the concept of 'available' iron.

5. The conclusion is drawn that the calculation of the total iron content of dietaries from standard food value tables is of very dubious value.

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A DIET AND PHYSIQUE SURVEY IN ASSAM, RURAL BENGAL, AND CALCUTTA.

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THERE appears to be an increasing appreciation in recent years of the importance from a public health point of view of the value of diet and physique surveys both as a means of assessing the standard of health of the people and of supplying information on the lines on which improvement, if necessary, should be concentrated. The present study is one of several which have been appearing from different laboratories in India and it is intended to discuss past and recent work in relation to the findings in this investigation rather than give a historical résumé.

The diet discussed in this paper covers three classes of families, viz., thirty-seven from industrial coolie workers in Assam, ten from an agricultural area in Bengal (Barasat), and six from a well-to-do Calcutta class including a boarding house. In Assam 1,591 children of both sexes and in Barasat 267 boys were examined in respect to height, weight, hand dynamometer grip, arm-chest-hip measurement (A. C. H. index of nutrition), signs of angular stomatitis, phrynoderma, Bitôt's spots, caries, malocclusion of teeth, and enlarged tonsils.

GENERAL CONDITIONS IN ASSAM

The subjects were of the coolie class, mostly Behari Hindus. They were for the most part domiciled in Assam and, unlike the jute-worker class, did not

send their children back to their original homes; in many cases indeed several generations had been resident in Assam. The majority had no dependants, not actually living with them. The children in most cases from the age of seven got employment in the industry. The average wages of the males were about Rs. 8 to Rs. 10 per month; females (who were employed for special work at a piece rate for about six months of the year) could earn somewhat more—Rs. 7 to Rs. 12 per month. The children of both sexes earned between Rs. 2 to Rs. 3 per month; wages were paid weekly. Every family had a house and plot of land for which they paid the employers a nominal rate. Those with larger wage-earning families could rent more and sometimes kept a cow. There was a small amount of indebtedness among themselves but no evidence of a tribute to foremen, etc., which is known to prevail in other industries. They were given a holiday once a week during which they appeared to be able to spend a certain amount on liquor. Their rice, dal, certain vegetables, and oil were purchased with cash, while green vegetables were supplied from their own plots. No milk was available for sale among the coolies; any milk consumed being derived from their own cow if they possessed one.

BARASAT AREA.

Those subjects were all of an agricultural middle class who worked on the land themselves and often employed additional labour as well. There were, in addition, two cobblers, a smith, and a pleader who also cultivated their own land; one was a relatively well-to-do zemindar. Practically all of them were dependent to a large extent on the land to supply their needs. Existence appeared to be rather hard to mouth and little money was handled. A certain amount of barter existed, fish, etc., being exchanged for soil products. In general there was a disinclination for manual work as being below their dignity. It appeared that more could have been made of the ground had they possessed the energy to work it. There was a certain amount of indebtedness.

CALCUTTA FAMILIES.

Those were all of a class earning between Rs. 200 to Rs. 500 per month capable of sending their children to the Hare School, Calcutta, and whose physique the writers have already discussed (Wilson, Ahmad and Mitra, 1937).

DIET SURVEY.

The survey was carried out by a house-to-house visit and covered a period of twenty-one days in Assam and seven in Barasat and Calcutta. The analysis of the diets covers the following: total protein, animal protein, total fat, animal fat, carbohydrates, total calories, per cent calories from cereals and pulses respectively, total minerals, calcium, phosphorus, Ca : P ratio, iron, vitamin A, carotene, vitamin B₁, flavin, vitamin B₆ (anti-dermatitis), and vitamin C. The food values were taken largely from the Health Bulletin No. 23 (1937) and from figures obtained from this Laboratory. The flavin figures were obtained from data from this Laboratory by biological assay. In this work there was often a difference

in the figures found by assay and chemical methods in favour of the former. As the basis of a diet survey is to assess the nutritional value of a food mixture in terms of its physiological effect we employed these—the assay—figures in preference. The values for the B₆ content were also obtained from this Laboratory. In many cases with this particular factor, we had to take the value of a food from a figure obtained from a similar foodstuff which had already been analysed. Much stress cannot, therefore, be laid on those values in this survey.

DISCUSSION OF DIETS.

The family co-efficients employed were those suggested by the Health Bulletin (*loc. cit.*) and the Stiebling scale (calories only) in addition (Sherman, 1937). As regards the other constituents—the consumption units (henceforth c.u.)—have been worked out from the bulletin, as the Stiebling scale is an expression of standard requirements rather than what is actually consumed. In recalculating, for instance, the actual amount of a food consumed by a child, it is obvious that the result will not vary much provided the same factor is used as was employed in obtaining the original c.u. In Assam there was every likelihood that the children received about their share—unselected—of the day's dietary.

It will be seen from Tables I, II, and III that the three groups differ quantitatively in almost every respect, Assam being the poorest and Calcutta the best, Barasat falling midway between.

CALORIES.

The following are the calorie consumptions calculated by the two standards for comparison :—

Groups.		Indian scale.	Stiebling scale.
Assam	..	2,101	2,036
Barasat	..	2,743	2,484
Calcutta	..	2,787	2,652

Caloric consumption per consumption unit.

On either standard, the Assam figure appears too low. The League of Nations (1936) puts forward a figure (for a temperate climate) of 2,400 calories

net plus 75 for each hour of moderate work; assuming that they do an eight-hour day this would mean a total requirement of 3,000 calories net. The Health Bulletin suggests a total daily intake for the average Indian of 2,600 calories. Of the three groups the Calcutta families alone come up to the Stiebling scale. This deficiency in calorie intake among the poorer class becomes noticeable when the actual children's allowance is calculated (Table V). An Assam boy of seven consumes 1,090 calories, while Rose (quoted by Sherman, 1937) recommends one of 1,600 to 2,100 per day. Even if it be assumed that an Assam boy of seven weighs as much as a European boy of five years, the intake at that age is assessed at 1,200 to 1,500 per day. A discussion of the heights and weights will tend to favour the conclusion that they appear to be receiving too little.

PROTEIN.

The consumption of this foodstuff ranges from 58.8 g. (Assam), 78.4 g. (Barasat) to 94.0 g. (Calcutta) per c.u. The percentage derived from animal sources likewise shows a similar trend, namely, 2.7 per cent (Assam), 10.8 per cent (Barasat), 33.0 per cent and 47.7 per cent (Calcutta). The well-to-do in Calcutta obviously by choice have a protein consumption which is quantitatively and qualitatively on a par with European standards, while at the other extreme the Assam workers are at a definitely low level in both those respects. The exact protein requirements, both quantitative and qualitative, necessary for the maintenance of health over a long period is still disputed. Leitch and Duckworth (1937) in a recent review conclude from the evidence available that probably 48.2 g. to 52 g. may represent the minimum at least for equilibrium over a long period. Many investigators have obtained nitrogen equilibrium on much less and, in view of the actual level of the endogenous (wear and tear) nitrogen metabolism—about 2 g. per day (12 g. protein)—there is every theoretical reason for a hypothesis that from 12 g. upwards of a good quality protein should be sufficient for an adult. It should be remembered, however, that equilibrium on such a low intake is dependent on (a) an adequate calorie intake containing a high percentage of carbohydrates to act as spacers of protein, and (b) a high biological value and co-efficient of absorption for the protein mixture fed.

CHILDREN'S INTAKE.

In Table V are calculated the actual protein intakes of Assam boys for each age from seven to twelve. These have been recalculated from the co-efficient given in the Health Bulletin. In Table VI are the figures obtained along with the standards recommended by the B. M. A. Committee (1933), Hawley, and Stiebling for comparison. The consumption of the Assam boys is obviously much below that recommended for their ages. A boy of twelve, for instance, receives 47 g., while the assessed requirement ranges from 80 g. to 87 g. per day. Holt, Courteney and Fales (quoted by Leitch and Duckworth, *loc. cit.*) recommend that the amount of calories derived from protein in the diet of a child should be 15 per cent. In the case of the Assam boys, owing to the low calorie intake, this works out at 10 per cent. In this respect their diet is perhaps qualitatively nearer the standard suggested but it should be borne in mind that with a low energy intake

less protein will be available for growth. Further, those boys are much below the better class Indian in height and weight and in this respect their intake of protein is perhaps at least better adjusted to their unfortunately diminished needs. It should be noted that there was practically no animal protein consumed by the Assam families. There was hence no chance that the young would get any selection from the diet, indeed there was little in the nature of a protein rich food for them to select with the exception of an occasional meal with meat in small quantity. Even on a weight basis those children are receiving less than has been recommended; as, for instance, 3 g. protein per kg. body-weight. An Assam boy of seven weighing 15 kg. should hence receive about 45 g. instead of which 29.4 g. are available.

A significant feature which probably illustrates physiological economy in nutrition is brought out by calculating the increase in body-weight in relation to the protein consumption. The following shows the average increase in body-weight in kg., the average percentage increase in weight per annum and the increase in weight per annum per gramme of protein consumed per kg. body-weight per day.

		Average rate of growth per annum, kg.	Average percentage increase in weight per annum.	Average rate of growth per g. protein consumed per kg. body- weight per day.
Assam	..	2.10	10.0	1.14
Barasat	..	2.05	8.6	0.93
Calcutta	..	2.46	9.3	1.07

It will be noted that the rate of growth (kg.) of the Assam boys is as great as those of Barasat who get a better diet. More striking is the rate of growth per annum per gramme of protein consumed per kg. body-weight per day; 1.14 Assam, 0.93 Barasat, and 1.07 Calcutta. Further the rate of growth as a percentage of the increase of body-weight is greatest in the Assam group (10 per cent). It would appear that in the Assam group the assimilation and utilization of protein and or food is better than in the other groups with a larger food intake. Certainly the growth impulse as judged by the percentage increase in weight is not wanting among the Assam children. A racial factor cannot be entirely excluded here but it seems possible that an insufficiency of

building materials or other factor has at an earlier age kept back the Assam group in relation to the Calcutta group by a year or two.

FAT.

The average intake of this food by the coolie and agricultural class in comparison to European standards is strikingly low, being 11.9 g. Assam, 19.7 g. Barasat, and 86.5 g. Calcutta per c.u. The problem of the fat requirements is as yet an unsolved and somewhat neglected problem. The fat consumption of the human race in general decreases as the equator is approached. This is in large measure due to what nature has provided in each region but attention should be drawn to the natural inclination of man in respect to this food. It will be noted that with an improvement in economic status the absolute increase in the fat intake is much greater than in the case of protein. The Calcutta families consumed eight times as much fat but only twice as much protein as the Assam coolies. A similar observation has been made in Europe by Cathcart and Murray (1932) where an increase in the food budget was associated with a larger fat consumption, often with little or no change in the protein level. In Calcutta, for instance, the natural inclination appears to demand a fat consumption of 86 g. of almost as high as is taken in Europe. In Table V is calculated the fat intake of the Assam coolie boys. The figures range from 5.9 g. to 9.5 g. per day, almost none of which is derived from animal sources. The former amount could easily be consumed by a laboratory rat and in view of the above observations it appears unlikely that a child can be considered properly nourished on such a low intake.

MINERALS.

Calcium is second to fat only in the degree to which it falls short, above all in Assam, of the European standards. The following are the intakes per c.u. of the three groups; 0.173 g. (Assam), 0.47 g. (Barasat), and 0.56 g. to 0.79 g. (Calcutta). Sherman (1937) recommends about 0.67 g. or with a safety factor of 50 per cent 1 g. for an adult and certainly this amount for a child. In Table V are shown the calculated allowances of the Assam boys at each age. A boy of seven, for instance, receives only 0.086 g. calcium per day. A calculation based on Table V brings out that from the ages seven to twelve 245 g. Ca would have been consumed in the diet. If 1.5 per cent Ca be accepted as the percentage of this element in the human body (Sherman, *loc. cit.*), it can be calculated from the weight of those boys that 147 g. Ca should have been added to the body in the five years. This addition could have been covered by a 60 per cent retention of the intake over those years, viz., 245 g. With the rather superior absolute rate of growth (kg. per year) of the Calcutta boys the retention should have been 186 g., i.e., involving 75 per cent of the intake. Apparently the low Ca intake is not incompatible with the theoretical requirements. Observations by Sherman (*loc. cit.*) show, however, that with a Ca intake of 0.3 g. per day about 45 per cent only is retained. In one experiment a child of 18 kg. body-weight, i.e., slightly more than the Assam boy of seven, had the capacity to retain 0.3 g. per day (intake nearly 1 g.) for the period of observation. This retention was much more than was even available (0.088 g.) in the coolie boys' diet. He

further states that the optimal retention is about 10 mg. per kg. body-weight per day. A point of criticism might be raised in relation to such a figure. Taking the average weight of all the well-to-do Calcutta boys between seven and twelve as 26.5 kg. and assuming a retention of 10 mg. Ca per kg. body-weight per day for the five years a figure of 483 g. Ca is arrived at. If the Ca is taken as 1.5 per cent of the body the weight increase over those five years should be 30 kg., i.e., about twice as much as the Calcutta, American, or British boys actually do gain over this period. In view of the findings recorded here it would appear that either the Ca content of the young is more than 1.5 per cent of the body-weight or a retention of 10 mg. per day would not continue if the observations were carried on long enough. Sherman's (*loc. cit.*) observations on rats do show, however, that the percentage of this element in the carcass of the rat may vary from 0.8 to 1.4 per cent according to the diet. If such a variability exists in the human subject it may be a factor in the cause of osteomalacia. The female endowed with a fragile or calcium poor bony tissue may not be able to decalcify further during pregnancy without affecting the mechanical stability of the skeleton. Work is definitely indicated as to the capacity of the Indian child to retain Ca on a typical diet. The evidence from this survey does, however, appear to show that the Ca intake is too low but that the standard set, namely, 1.0 g. per day, may not be necessary for normal growth.

PHOSPHORUS.

The phosphorus intake varies from 1.67 g. (Assam) to 2.02 g. (Calcutta) per c.u. These figures do not appear to require comment except to point out that the average Ca : P ratio is 1 : 9.6 (Assam) and 1 : 2.6 (Calcutta). The former ratio is at least favourable to the absorption of Ca and unfavourable to the production of rickets. A ratio of 1 : 2 in the diet is considered to be the optimum for the two minerals.

IRON.

This element is of special interest in that there is evidence of a certain amount of hypochromic and/or iron deficient anæmia (personal communication) in the Assam group. The intake ranges from 18.7 mg. (Assam), 27 mg. (Barasat) to 33 mg. (Calcutta) per c.u. In Assam probably only about 50 to 75 per cent is in an available form. As regards the normal iron requirements opinions vary. Sherman (*loc. cit.*) has estimated from a review of the literature that about 12 mg. is a generous allowance for an adult. He quotes experiments to show that the retention of iron is better on a bread and milk than on a bread and egg-white diet: the difference is, however, small. For children an allowance of 8 mg. to 12 mg. per day is advocated which is certainly more than the young coolies would be receiving. It should be mentioned, however, that the water in Assam is rich in iron, but how much is in an available form is unknown. Orten, Smith and Mendel (1936) have further shown that rats fed on a diet low in total minerals and iron develop a polycythæmia with a low hæmoglobin percentage which can be cured more effectively by the addition of extra calcium than by iron. Similar observations on the effect of calcium on iron retention in the human subject have been

made by Davidson, Fullerton, Howie, Croll and Orr (1933). It should be noted that the total mineral intake in the Assam group is half that of the Calcutta one. Such observations suggest that the problem of iron and associated elements such as calcium may be related to this type of anæmia.

VITAMIN A AND CAROTENE.

The consumption of vitamin A plus carotene per c.u. varies from 1,000 γ (Assam) to over 7,500 γ (Calcutta). In the former group this is derived almost entirely from the carotene of the green vegetables, while in the latter some 800 γ are from pre-formed sources of vitamin A. According to Guilbert and Hart (1935) the requirements of vitamin A are in proportion to body-weight and are assessed at 20 γ per kg. From Table V it will be seen that the Assam boy of seven receives a total of 497 γ which is fully 30 γ per kg. According to Aykroyd and Krishnan (1936) clinical signs of deficiency were observed where the intake amounted to about 828 γ per c.u. As will be noted later (Table VIII-A) the incidence of xerophthalmia 15 per cent and Bitôt's spots 2·4 per cent in Assam would indicate that 1,000 γ is barely sufficient to prevent the appearance of the disease which certainly tends to discountenance the assertion that 20 γ per kg. body-weight at least in association with the other constituents of this particular diet is adequate. Wilson, Gupta and Ahmad (1937) have shown that the absorption of carotene in the human subject is better on a fat rich diet, a fact which has obvious bearings on this survey. Further, Wilson, Ahmad and Mazumdar (1936) have shown in rats that a diet containing meat may favour the transformation of carotene into vitamin A in the liver. Moreover, the quantitative observations recorded here fall closely into line with those of Aykroyd *et al.* in South India.

VITAMIN C.

The intake of ascorbic acid varies from 26 mg. (Assam), 86 mg. (Barasat) to 133 mg. (Calcutta). Sherman quotes observations of Euler and Malmberg who found in Sweden symptoms of scurvy when the consumption fell to between 5 mg. and 11 mg. per day. Gothlin (1934) found the minimum amount of vitamin C to prevent incipient scurvy to be between 19 mg. and 27 mg. per day. When it is considered that the vitamin C among the Assam workers was in the form of vegetables which were cooked, it is unlikely that their tissues were saturated with this substance. No obvious signs of vitamin-C deficiency were, however, observed among the three groups.

VITAMIN B₁.

The intake of vitamin B₁ ranges from 624 I. U. (Assam), 777 I. U. (Barasat) to 686 I. U. (Calcutta) per c.u. Cowgill (1934) has shown that the vitamin-B₁ requirements are in proportion to the energy consumption but exact figures for the human subject depend on clinical observations along with an analysis of their diets. Of two sets of families studied in Newfoundland by Aykroyd (1930) one group only of which had beri-beri, the vitamin-B₁ intakes were 27 I. U. (beri-beri)

and 391 I. U. (beri-beri free) re-calculated from Cowgill's estimation and expressed in International Units. If we accept 400 as being adequate the diets discussed here appear to be satisfactory in this respect. The reason for the relatively high figure in Assam is due to the consumption of unmilled rice.

VITAMIN B₆ (ANTI-DERMATITIS FACTOR).

The values in rat units vary from 279 (Assam), 326 (Barasat) to 363 (Calcutta). The significance of this factor for the human subject is as yet obscure. The numerical figure, however, probably gives an index of the amount of some of the B group of vitamins which appear to be necessary for human nutrition. In the light of Aykroyd and Krishnan's (1936) work on the effect of yeast in curing angular stomatitis in children it is possible that either this substance or one of similar properties and closely associated with it (filtrate factor) may be involved. Angular stomatitis was observed in only two cases in Assam. None were observed in the other groups.

The significance of this substance in human nutrition has yet to be elucidated. As it is present in human tissues it is likely to be an essential dietary constituent although in practice a deficiency may never occur. The amount consumed by the three groups varied between 1.0 mg. and 1.5 mg. per c.u.

PHYSIQUE AND CLINICAL SURVEY.

The most striking difference between the three groups are those of height and weight (Table VII). The well-to-do Calcutta boy is, at the age of twelve, seventeen pounds heavier than his coolie brother. The difference is still more marked in the case of the girls. The boys of the Barasat agricultural class fell midway between. The same features are to be noted for the heights in the three groups. As has already been pointed out (Table VII, Charts 1 and 2) the growth of the coolie boy expressed as a percentage increase in body-weight per year is actually greater than in either of the other classes.

DYNAMOMETER GRIP.

The hand grip shows corresponding differences for the three groups when each age is compared. The significance of this finding is somewhat altered, however, when the grip per pound body-weight is calculated. The figures are seen to be distinctly higher in the two groups accustomed to manual labour, namely, Assam and Barasat. This may be due in part to the greater amount of muscle tissue in relation to body-weight—and to their being accustomed to manual labour particularly in Assam where all the children were lighter in weight—in comparison to the better off boys in Calcutta. The slightly greater superiority of the Barasat boys—actually probably still larger as they had a certain amount of adipose covering—may be due to a more powerful muscle tissue secondary to a better diet relatively to the Assam group.

CHART 1.
Height-Age.

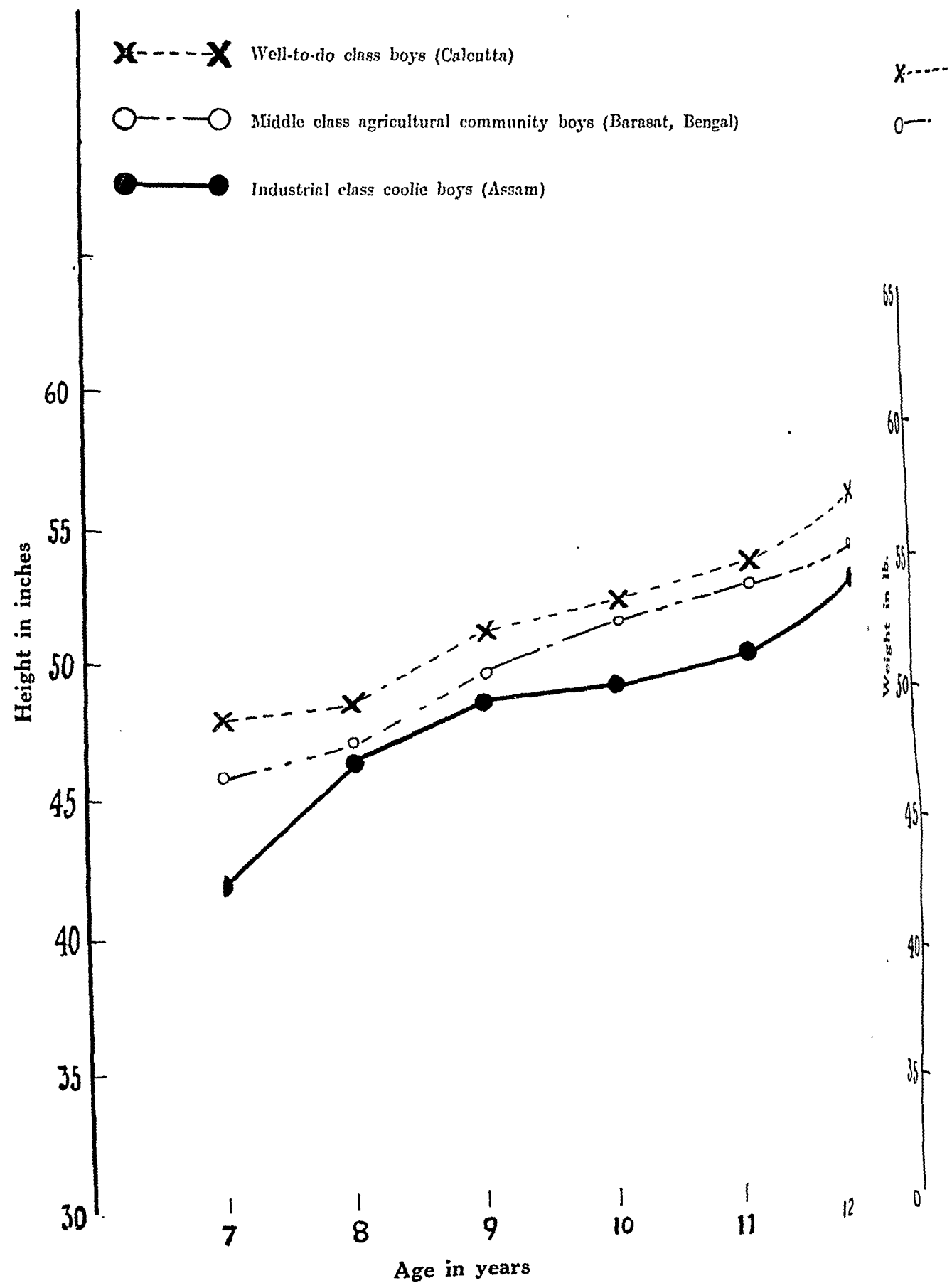
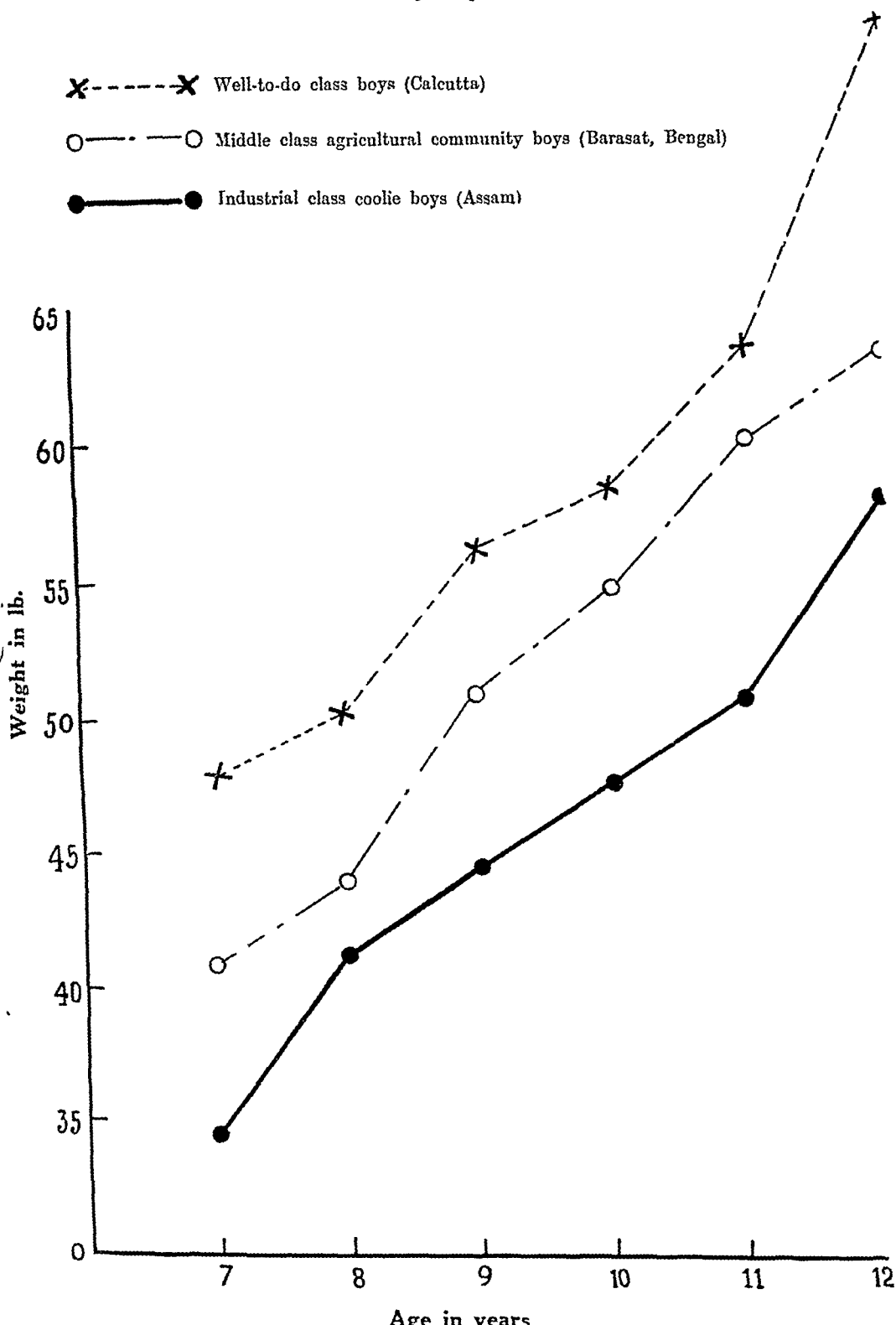


CHART 2.
Weight-Age.



SYMPTOMS OF DEFICIENCY.

The boys in Assam may be compared to children examined by Aykroyd and Krishnan (1936) in South India (group III, Mayanur district) as they were approximately of the same weight. In Assam only were definite cases of angular stomatitis observed, 2 boys (0·2 per cent), while the figure for the Mayanur children was 6·6 per cent, of phrynoderma four cases (0·2 per cent) including both sexes were observed and one case (0·2 per cent) in South India. The small incidence of phrynoderma associated with a low fat and carotene intake in Assam would appear to indicate that those two dietary factors are not involved in this condition. It should be mentioned, however, that the skin of the children in this area was dry both in appearance and touch. The incidence of xerophthalmia and Bitôt's spots was somewhat higher in Assam, viz., xerophthalmia 15·0 per cent boys and 7·9 per cent girls, and Bitôt's spots 2·4 per cent boys and 0·8 per cent girls. The incidence of Bitôt's spots for the group in South India was 3·8 per cent. The carotene intakes in Assam and South India were 1,017 γ (including vitamin A) and 828 γ , respectively, per c.u. This would tend to confirm the observation that 700 γ per c.u. is inadequate and that 1,000 γ per c.u. is about the threshold value for appearance of signs of deficiency, at least when taken as part of the diet under discussion.

The incidence of caries showed no correlation with diet. It was found to be lowest in Assam (8·7 per cent girls and 11·0 per cent boys) and largest in Barasat (27·9 per cent boys). The incidence of enlarged tonsils, however, showed a negative correlation with the poverty of the diet. It was least in Assam (12·8 per cent boys and 19·6 per cent girls, Barasat 28·9 per cent boys, and Calcutta 42·3 per cent boys and 44 per cent girls). The incidence of malocclusion of teeth, on the other hand, showed a distinct correlation with economic status as was noted by Nicholls (1936) in Ceylon (Assam 23·2 per cent boys and 26·7 per cent girls, Barasat 14·9 per cent boys, and Calcutta 8·0 per cent boys and 9·3 per cent girls). There appeared to be no correlation between caries and malocclusion of teeth. The low incidence of caries in the coolies would appear to indicate that calcium is not an important factor in the production of this condition. The higher incidence among the well-to-do may be associated with the consumption of sweetmeats.

A. C. H. INDEX.

The figures for the selection by the index in Assam are higher than any observed by the writers in their previous survey (Table VIII-B) (Wilson, Ahmad and Mitra, *loc. cit.*). The selection is distinctly correlated with economic status; Assam 38·7 per cent boys and 36·9 per cent girls, Barasat 33·8 per cent boys, and Calcutta 16·3 per cent boys and 18·8 per cent girls. Previous observations have shown that there is no correlation between the selection by the index and the heights, weights and incidence of caries or enlarged tonsils. It will be seen, however, that there is a distinct correlation between the signs of xerophthalmia and the selection by the index. In Assam the selection figure for all the boys was 38·7 per cent, while of these only those who were suffering from xerophthalmia are taken the percentage is 47·6 per cent. A glance at the table will show that the index is

still more effective in selecting Bitôt's spots—the more advanced stage. It is likely that many selected by the index are just on the border line of showing symptoms. It will be noted that the index does not select cases of malocclusion of teeth; if anything it tends to exclude them.

DISCUSSION OF PHYSIQUE.

On the combined evidence given by height and weight measurements, A. C. H. index, and clinical signs of deficiency disease, the Assam coolies and to a certain extent the Barasat boys appear to be suffering from a certain degree of malnutrition. Is the diet defective qualitatively, quantitatively, or both? The signs of deficiency disease clearly show that there is certainly a qualitative defect in respect of substances containing carotene such as leafy vegetables. The relationship between the low body-weight on the one hand and the low energy, protein and calcium consumption on the other may not be so clear. Aykroyd and Krishnan's (1937) work certainly suggests, however, that the latter may be the most important conditioning factor. As has been shown, however, the actual rate of growth is above that of the well-to-do class in Calcutta. The possibility of a racial difference to account for the smaller physique can not be entirely excluded although against this there are observations (Wilson, Ahmad and Mitra, *loc. cit.*) which show that in Calcutta marked differences in weight in the same community are found to be associated with economic status. Unfortunately we have no figures in Assam for the better-off classes. The Assam coolies do not appear, however, to lack vitality as measured by their grip: weight ratio and relative rate of growth.

IMPROVEMENT IN DIET.

In Table IV are given analyses in terms of the foodstuffs actually consumed by the three groups. As the economic status is improved, it will be noted that the consumption of milk, animal foods, leafy vegetables, dals and fruits is increased. On the findings of the clinical and diet surveys the consumption of foods rich in carotene such as leafy vegetables should be increased. This deficiency could be relatively easily remedied by encouraging the cultivation of such leafy vegetable as amaranth and drumstick leaves. An increased consumption of dals should also be within the means of both the Assam and Barasat groups. Experience, however, all over the world in recent years has shown that milk is probably the best supplement to a poor diet especially in the young. This, however, is an expensive luxury for most. A cheap and possible substitute, at least as an expedient, is suggested by the recent observations of Aykroyd and Krishnan (1937). They have shown that the addition of calcium lactate or yeast to a poor Madrassi diet is effective in promoting the growth of rats. Cystine, casein, and flavin were found to be without effect. In view of the poverty of the Assam diet in calcium this addition would appear to be a cheap substitute to try until such time as milk can be made available in adequate amount. Further, the addition of extra dal might help to improve the diet in substances containing the factors present in yeast. One point of interest might be mentioned here. The fact that the Assam coolies appear to show an active growth rate in contrast to rats fed on the

same diet even with extra fat (unpublished) would appear to show either that rat requirements differ from those of humans or the children are actually getting more calcium or yeast factor than is assessed in their diet. Children have been observed elsewhere, even in Calcutta schools, to eat plaster from walls, etc. Calcium in the drinking water and the chewing of pan as they grow older are obvious sources of this element.

SUMMARY.

1. A diet and physical survey has been carried out in three groups of families of different economic class, namely, Assam industrial workers, rural Bengal (Barasat) agricultural class, and well-to-do Calcutta families.

2. The diet of the well-to-do appears to approximate in most respects to the European standards. The diet of the Assam coolie class is poor in total calories, total and animal protein, total and animal fat, calcium, and carotene.

3. The heights and weights of the Assam children from seven to twelve years of age are much lower than those of the other groups. A similar finding has been found in Assam in respect to the hand dynamometer grip.

4. The actual rate of growth expressed as a percentage increase in body-weight per year and the dynamometer grip per lb. body-weight (age neglected) are greater in the Assam than the Calcutta children. A certain percentage of the Assam children had xerophthalmia and Bitôt's spots with a carotene plus vitamin-A intake of 1,000 γ per c.u. It is likely that the figure is near the threshold value for the appearance of signs of vitamin-A deficiency.

5. A few cases of angular stomatitis and phrynoderma were observed among the Assam children.

6. The incidence of caries is greatest among the well-to-do and least among the poor Assam coolies. The case is just the reverse with the incidence of malocclusion of teeth.

7. The selection of children by the A. C. H. index was twice as high in Assam than in the Calcutta group.

8. The Assam families have certainly a qualitative and probably a quantitatively deficient diet.

9. The diet could be improved up to a point and without much extra expenditure by the additional consumption of leafy vegetables and extra dal.

10. It is likely that the Assam and Barasat children in spite of a possible racial factor are below the optimum in physique and health due largely to malnutrition.

ACKNOWLEDGMENT.

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Diet survey of thirty-seven families of industrial workers (Assam) : intake of proximate

Serial number of family.	Number of persons.	Number of consumption units.	PROTEIN (G.).		FAT (G.).		CARBO-HYDRATE (G.).	CALORIES	
			Total protein.	Percentage of animal protein.	Total fat.	Percentage of animal fat.		Total calories.	Percentage from cereals.
1	2	1.6	92.2	6.1	16.7	11.3	575	2,818	79.1
2	9	6.4	63.1	0.8	10.5	0.4	526	2,451	92.4
3	6	4.3	46.7	6.4	7.9	24.1	375	1,774	92.0
4	3	2.4	71.7	13.5	25.0	31.9	570	2,829	83.3
5	3	2.0	51.9	0	10.4	0	392	1,870	89.3
6	7	3.9	48.4	0	10.4	0	398	1,880	90.2
7	1	0.8	40.5	0	12.5	0	319	1,575	85.8
8	3	2.8	35.9	0	13.4	0	292	1,434	84.8
9	2	1.8	49.4	0	6.9	0	358	1,814	88.4
10	3	2.1	49.2	0	10.0	0	421	1,972	90.1
11	4	3.3	58.8	1.1	12.18	0.4	490	2,338	91.7
12	7	4.2	21.4	6.8	10.2	14.3	179.5	901	88.0
13	3	2.3	72.8	5.5	8.0	1.0	583.0	2,728	91.1
14	4	2.3	55.6	1.5	10.6	6.3	441.0	2,083	89.9
15	2	1.8	66.6	5.0	12.5	2.4	524	2,276	89.2
16	3	1.8	81.0	0	17.0	0	661	3,126	90.0
17	5	3.6	93.0	7.1	15.7	0.3	686	3,141	87.8
18	4	2.7	60.0	1.3	15.2	0.3	507	2,406	89.1
19	4	3.5	40.0	1.5	6.5	0.7	348	1,613	92.9
20	3	2.2	76.2	2.8	12.0	1.0	579	2,715	87.8
21	4	3.0	61.1	4.3	8.8	7.4	509	2,367	92.6
22	1	0.8	97.1	0	24.4	0	702	3,419	81.1
23	1	1.0	81.2	0	11.6	0	572	2,721	82.5
24	6	3.5	54.7	0.6	7.3	0.2	471	2,169	94.1
25	3	1.5	46.4	1.5	7.8	0.6	396	1,840	94.0
26	4	2.9	49.7	4.4	9.1	1.0	398	1,874	91.1
27	3	2.2	54.1	8.5	18.7	10.7	376	1,877	82.1
28	2	1.8	33.0	0	6.1	0	276	1,295	90.6
29	1	0.8	72.3	0	13.8	0	550	2,846	86.0
30	3	2.1	78.3	6.5	6.3	0.6	395	1,887	95.9
31	2	1.6	54.3	0	12.0	0	302	1,457	89.8
32	1	0.8	66.1	0	23.8	0	558	2,671	82.9
33	4	2.4	61.5	4.4	16.5	1.2	471	2,253	87.3
34	2	1.6	72.6	2.8	13.5	1.1	561	2,649	89.2
35	7	5.1	39.2	5.7	5.0	0.3	327	1,509	93.2
36	4	2.9	42.4	3.7	7.7	1.4	347	1,631	92.5
37	5	3.8	60.2	0	7.2	0	497	2,293	92.4
TOTAL ..	131	93.7	2,178.6	101.8	443.8	119.0	16,942	80,702	3,290.2
AVERAGE	3.5	2.53	58.8	2.7	11.99	3.2	458	2,181	88.9

principles, calories, minerals, and vitamins per consumption unit per day.

MINERAL MATTER (G.).				VITAMINS.						
Total mineral.	Calcium.	Phosphorus.	Ratio Ca: P.	Iron, mg.	Vitamin A (γ).	Carotene (γ).	Vitamin B ₁ (International Units).	Flavin, mg.	Vitamin B ₆ (rat units).	Vitamin C (ascorbic acid), mg.
9.50	0.32	2.21	1: 6.9	24.80	8.4	1,244	841	1.520	365	75
7.29	0.14	1.89	1: 13.5	20.73	0.6	585	708	1.050	312	19
5.07	0.09	1.38	1: 15.3	14.46	5.0	193	504	0.846	234	9
9.9	0.39	2.29	1: 5.8	24.89	373.0	739	804	1.487	388	49
5.55	0.13	1.42	1: 10.9	14.67	0	701	543	0.923	237	9
5.30	0.09	1.42	1: 15.8	14.78	0	149	527	0.897	231	11
6.00	0.24	1.19	1: 5.0	16.27	0	3,004	456	0.780	201	35
4.36	0.12	1.04	1: 3.7	12.06	0	1,584	393	0.687	175	23
5.80	0.15	1.40	1: 11.1	15.60	0	1,345	545	0.905	240	25
6.02	0.10	1.47	1: 14.7	16.25	0	339	563	0.940	243	20
7.05	0.19	1.81	1: 9.5	20.60	0.8	2,527	655	1.123	302	24
2.58	0.10	0.68	1: 6.8	7.15	75.0	843	234	0.415	102	8
7.99	0.19	2.13	1: 11.2	22.58	8.0	1,625	773	1.314	257	18
7.17	0.25	1.56	1: 6.2	19.60	5.2	877	590	1.017	268	21
7.85	0.17	1.95	1: 11.4	21.53	4.0	1,161	711	1.171	325	12
10.40	0.22	2.39	1: 10.9	29.40	0	1,657	905	1.534	403	47
12.10	0.45	2.68	1: 5.9	30.90	3,420	663	1,003	1.794	420	35
7.27	0.17	1.80	1: 10.5	20.50	0.9	606	667	1.153	313	41
5.08	0.09	1.27	1: 13.0	14.00	0.7	360	453	0.772	215	28
8.60	0.18	2.10	1: 11.6	22.80	3.0	534	783	1.371	350	35
6.61	0.13	1.80	1: 13.3	19.00	3.3	454	672	1.120	313	12
10.13	0.25	2.53	1: 10.1	25.90	0	313	1,027	1.762	431	41
8.42	0.23	2.09	1: 9.1	21.60	0	1,077	805	1.439	342	45
6.70	0.13	1.68	1: 12.9	18.87	0.4	1,014	618	1.033	284	19
5.50	0.15	1.42	1: 9.6	16.40	0.8	1,940	517	1.084	237	11
5.71	0.12	1.46	1: 12.2	16.30	3.1	473	532	0.905	260	72
5.60	0.20	1.36	1: 6.8	14.80	5.6	1,037	524	0.909	234	32
3.89	0.11	1.00	1: 9.0	12.90	0	1,016	363	0.624	163	20
8.45	0.19	2.06	1: 10.0	24.80	0	710	816	1.430	368	66
5.32	0.13	1.39	1: 10.6	16.00	0.6	1,285	504	0.844	233	20
4.40	0.11	1.08	1: 9.8	12.80	0	1,539	397	0.669	183	20
8.46	0.18	1.99	1: 11.0	24.70	0	675	722	1.288	333	62
7.14	0.14	1.76	1: 12.5	19.00	0.3	441	636	1.099	290	10
7.70	0.19	2.04	1: 10.7	21.80	2.3	1,114	746	1.270	227	18
4.27	0.8	1.16	1: 13.6	12.27	0.3	465	468	0.723	189	3
5.15	0.15	1.25	1: 8.3	14.40	1.8	1,140	455	0.772	204	8
6.52	0.16	1.82	1: 11.3	19.03	0	1,397	652	1.091	287	10
250.85	6.43	61.97	1: 9.63	694.84	845.1	36,826	23,112	39.769	10,359	963
6.77	0.173	1.67	1: 9.63	18.77	22.8	995	624	1.074	279	26

TABLE

Diet survey of ten families at Barasat (Bengal) : intake of proximal:

Serial number of family.	Number of persons.	Number of consumption units.	PROTEIN (G.).		FAT (G.).		CARBO-HYDRATE (G.).	CALORIES.		
			Total protein.	Percentage of animal protein.	Total fat.	Percentage of animal fat.	Total.	Total calories.	Percentage from cereals.	Percentage from pulses.
1	11	8.0	107.0	4.1	17.3	1.9	971	4,543	82.4	76
2	7	5.4	54.5	6.3	17.1	0.3	408	2,006	83.6	46
3	5	2.9	50.6	5.4	10.6	2.6	337	1,647	81.3	106
4	11	7.7	68.0	11.4	11.2	13.4	582	2,684	79.9	21
5	12	8.4	154.2	41.6	65.1	44.4	830	3,921	65.5	21
6	7	4.7	72.5	16.9	19.1	37.7	550	2,668	79.7	13
7	2	1.8	88.9	0	24.2	0	571	2,859	65.1	181
8	10	8.5	75.3	1.4	9.3	11.5	508	2,738	85.1	59
9	9	6.9	49.5	15.9	12.7	29.6	369	2,033	71.1	23
10	5	4.5	63.6	5.2	10.7	1.4	496	2,333	86.0	43
TOTAL ..	79	58.8	784.1	108.2	197.2	142.8	5,622	27,432	779.7	594
AVERAGE	7.9	5.9	78.4	10.8	19.7	14.3	562	2,743	78.0	59

TABLE

Diet survey of five families (well-to-do class) and a boarding house at Calcutta (Bengal) : intake

Serial number of family.	Number of persons.	Number of consumption units.	PROTEIN (G.).		FAT (G.).		CARBO-HYDRATE (G.).	CALORIES.		
			Total protein.	Percentage of animal protein.	Total fat.	Percentage of animal fat.	Total.	Total calories.	Percentage from cereals.	Percentage from pulses.
1	9	5.9	89.8	40.5	55.3	35.1	431	2,680	55.7	23
2	10	7.3	84.5	37.8	32.0	27.3	376	2,137	64.1	88
3	10	8.7	94.7	69.0	85.2	66.5	404	2,860	43.2	35
4	10	8.8	112.3	49.5	177.6	60.7	404	3,667	34.5	0.5
5	9	7.8	88.7	41.6	72.5	44.9	394	2,592	45.0	64
TOTAL ..	48	38.5	470.0	238.4	432.6	234.5	2,009	13,936	242.5	22.0
AVERAGE	9.6	7.7	94.0	47.7	86.5	46.9	402	2,787	48.5	44
A boarding house.	20	20	82.6	33.0	116.2	12.5	423	3,077	44.5	4.8

II.

principles, calories, minerals, and vitamins per consumption unit per day.

MINERAL MATTER (G.).				VITAMINS.				Flavin, mg.	Vitamin B ₆ (rat units).	Vitamin C (ascorbic acid), mg.
Total mineral.	Calcium.	Phosphorus.	Ratio Ca : P.	Iron, mg.	Vitamin A (γ).	Carotene (γ).	Vitamin B ₁ (International Units).			
16.90	0.76	3.72	1 : 4.8	50.4	100	6,976	1,340	2.370	610	147
6.65	0.27	1.67	1 : 6.0	21.3	0	2,056	557	0.984	263	74
6.68	0.48	1.34	1 : 2.8	21.0	141	4,431	497	0.920	209	119
8.10	0.26	1.98	1 : 7.6	21.2	61	3,317	704	1.275	329	49
12.30	0.94	3.60	1 : 3.8	44.0	58	7,438	1,110	2.119	615	61
10.90	0.53	2.16	1 : 4.0	24.7	35	2,700	754	1.411	354	84
10.20	0.26	1.96	1 : 7.5	20.0	0	977	838	1.610	369	36
10.26	0.56	2.16	1 : 3.8	32.8	53	4,325	806	1.490	356	153
6.33	0.21	1.47	1 : 7.0	14.5	177	137?	499	0.932	234	22
8.79	0.46	1.83	1 : 4.0	19.9	2	3,453	664	1.112	292	117.
27.12	4.73	21.89	..	269.8	627	35,810	7,769	14.223	3,631	862
9.71	0.47	2.19	1 : 4.6	27.0	63	3,581	767	1.422	363	86

II.

f proximate principles, calories, minerals, and vitamins per consumption unit per day.

MINERAL MATTER (G.).				VITAMINS.				Flavin, mg.	Vitamin B ₆ (rat units).	Vitamin C (ascorbic acid), mg.
Total mineral.	Calcium.	Phosphorus.	Ratio Ca : P.	Iron, mg.	Vitamin A (γ).	Carotene (γ).	Vitamin B ₁ (International Units).			
12.29	0.87	2.00	1 : 2.3	34.29	472	9,456	602	1.369	326	180
9.95	0.61	1.79	1 : 3.0	22.99	409	3,680	567	1.205	289	87
13.90	0.89	2.30	1 : 2.5	32.93	854	7,807	923	1.304	366	151
11.11	0.71	1.99	1 : 2.8	21.48	1,451	4,076	597	1.316	332	90
13.23	0.86	2.04	1 : 2.4	30.26	910	9,024	643	1.191	318	157
60.48	3.94	10.12	..	141.95	4,096	34,043	3,432	6.385	1,631	665
12.09	0.79	2.02	1 : 2.6	28.39	819	6,809	686	1.277	326	133
12.60	0.56	1.89	1 : 3.3	33.46	147	9,016	602	1.337	357	224

TABLE IV.

*Consumption of different foodstuffs per consumption unit per day
in Assam, Barasat, and Calcutta families.*

Major articles of food.			CONSUMPTION PER DAY PER C. U.			A boarding house, Calcutta.
			Assam.	Barasat.	Calcutta.	
			Oz.	Oz.	Oz.	Oz.
Rice	19·0	23·7	10·2	13·6
Atta	0	6	3·1	0·1
Dals	1·0	1·4	1·5	1·5
Leafy vegetables	0·2	0·8	0·7	2·7
Non-leafy vegetables	3·4	9·2	11·6	17·3
Fish, eggs, and meat	0·2	1·8	5·6	4·3
Milk	0·5	3·1	11·0	1·2
Mustard oil	0·3	0·6	2·2	3·8
Fruits	0	0·6	3·3	1·3

TABLE V.

Calculated intakes of certain food principles by Assam boys at different ages.

Age group.	Calories.	Protein.	Fat.	Ca.	P.	Fe.	Carotene.
		g.	g.	g.	g.	mg.	g.
7	1,090	29.4	5.9	0.086	0.83	9.38	497
8	1,308	35.2	7.1	0.103	1.00	11.26	597
9	1,308	35.2	7.1	0.103	1.00	11.26	597
10	1,526	41.1	8.3	0.121	1.16	13.13	696
11	1,526	41.1	8.3	0.121	1.16	13.13	696
12	1,744	47.0	9.5	0.138	1.33	15.01	796

TABLE VI.

The protein intake of Assam, Barasat, and Calcutta boys in relation to different standard requirements.

Age group.	Assam.	Barasat.	Calcutta.	B. M. A.	Hawley.	Stiebling.
	g.	g.	g.	g.	g.	g.
7	29.4	39.2	47.0	60	67	55
8	35.2	45.0	56.4	60	67	65
9	35.2	45.0	56.4	70	67	65
10	41.1	55.0	65.8	70	67	75
11	41.1	55.0	65.8	80	87	75
12	47.0	62.9	75.2	80	87	75

TABLE VII.

Height, weight and dynamometer grip of the Assam, Barasat, and Calcutta children.

Age group.	HEIGHT (inches).				WEIGHT (lb.).				DYNAMOMETER GRIP.				DYNAMOMETER GRIP PER POUND OF BODY-WEIGHT.			
	ASSAM.		BARASAT BOYS.		ASSAM.		BARASAT BOYS.		CALCUTTA.		ASSAM.		BARASAT BOYS.		CALCUTTA.	
	Boys.	Girls.	Boys.	Girls.	Boys.	Girls.	Boys.	Girls.	Boys.	Girls.	Boys.	Girls.	Boys.	Girls.	Boys.	Girls.
5-6	..	39.1	44.2	43.0	..	28.6	38.1	..	38.2	10.2	..	0.36	..	0.27
7	42.8	42.4	45.8	46.6	48.1	34.8	40.8	47.7	43.2	47.7	34.8	12.5	0.29	0.36	0.40	0.29
8	46.7	45.9	47.2	48.4	48.5	41.3	43.9	50.2	50.0	50.2	41.3	15.8	0.38	0.38	0.37	0.31
9	48.5	48.3	49.8	50.7	51.3	44.5	50.9	56.2	54.9	56.2	44.5	18.2	0.36	0.39	0.37	0.33
10	49.4	48.8	51.7	53.8	52.4	47.2	54.7	58.3	68.9	58.3	47.2	23.4	0.43	0.42	0.37	0.33
11	50.5	49.0	53.1	55.8	53.8	50.6	59.8	63.4	74.3	63.4	50.6	24.3	0.41	0.43	0.39	0.33
12	53.4	52.7	54.7	57.2	56.5	57.8	63.1	75.0	79.0	75.0	57.8	27.2	0.45	0.43	0.38	0.34
13	59.1	59.0	..	66.2	77.4	..	91.0	30.2	..	0.47	..	0.33
14	..	55.5	60.6	59.8	87.4	..	95.2	32.1	..	0.47	..	0.34
15	66.7	60.3	92.0	..	99.7	34.8	..	0.49	..	0.35
16	63.1	60.6	102.8	..	94.8	33.8	..	0.53	..	0.35
17	65.3	59.7	106.9	..	110.7	34.8	..	0.56	..	0.31

Total number examined :—

Assam (coolie class)	..	{ Boys 836 Girls 755
Barasat (agricultural middle class)	..	{ Boys 267 Girls 331
Calcutta (well-to-do class)	{ Boys 385 Girls 385

TABLE VIII-A.

Incidence of different clinical conditions observed. Number of cases and percentage.

Locality.	Children.	Total number examined.	Angular stomatitis.	Phryno-derma.	Xerophthalmia.	Bitot's spots.	Caries.	Malocclusion of teeth.	Enlarged tonsils.
Assam ..	Boys ..	927	2	3	139 (15.0 per cent).	22 (2.4 per cent).	102 (11.0 per cent)	216 (23.2 per cent).	119 (12.8 per cent).
	Girls ..	758	0	1	60 (7.9 per cent).	6 (0.8 per cent).	65 (8.7 ")	157 (20.7 per cent).	149 (19.6 per cent).
Barasat	Boys ..	294	*	*	*	*	82 (27.9 ")	44 (14.9 per cent).	85 (28.9 per cent).
Calcutta	Boys ..	298	*	*	*	*	48 out of 184 cases were examined for caries (26.1 per cent).	24 (8.0 per cent).	133 (44.6 per cent).
	Girls ..	385	*	*	*	*	74 (19.2 per cent)	36 (9.3 per cent).	163 (42.3 per cent).

* A few doubtful cases only were observed.

A DIET SURVEY OF FIFTY-ONE FAMILIES OF FEROZEPORE, PUNJAB.

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A NUTRITION survey of 1,250 school children of Ferozepore was carried out a year ago (Wilson, Ahmad and Mitra, 1937). At the same time a study of the diet of some representative families of the area was undertaken with a view to correlate, if possible, the state of nutrition of the people with the quality of their food. This was done in the early summer months of April and May 1936. A year later in the same months (April and May 1937) the work was extended to 51 families with a total population of 245 persons. These families were representative of the homes from which the school children, as reported previously, came.

The data were collected with the help of trained volunteers, the teachers of the local Government High School. Only such families were selected as were willing to co-operate in the investigation, and generally speaking the volunteer was known to the families that he visited. The records of the quantity and kind of foodstuffs consumed were obtained by two visits daily at the time of the principal meals and were entered on printed sheets, which were examined each day by one of the authors.

As the foods consumed were similar from day to day, it was not considered necessary to continue the investigation longer than 7 to 10 days. It may, however, be profitable to repeat the investigation in other seasons of the year when different types of foodstuffs are available.

According to their economic status the families can be placed only in two divisions: (i) 43 families of the lower middle class consisting of shopkeepers, school teachers, and other skilled workers with a monthly income varying from Rs. 40 to Rs. 100; and (ii) 8 families of sweepers or unskilled workers with a monthly income of Rs. 15 to Rs. 25. Division (i) includes the principal communities of the area—Hindus, Mohammedans, and Sikhs. As it is generally presumed that there are differences in the dietary habits of the different communities, the families have been divided into the following four groups for the purpose of this study:—

Group	I 16 families of Hindus.
„	II 19 families of Mohammedans.
„	III 8 families of Sikhs.
„	IV 8 families of Sweepers.

The information collected consisted of the quantity of raw edible portion of each kind of foodstuff consumed by the family from day to day. No allowance has been made for any difference in the nutritional value of foods after cooking or for waste. It is presumed that there was no waste since it is customary to eat the unconsumed food at the next meal. Allowance has, however, been made for the food given to domestic animals or guests.

The data have been analysed for total protein, fat, carbohydrates, animal protein, animal fat, ash, calcium, phosphorus, total calories, and percentage of calories from protein, fat, carbohydrates, and cereals. The results are expressed in terms of consumption units per day. To reduce the data into consumption units the International scale of family co-efficients has been used (League of Nations' Health Organization, 1932). These data are shown in Table I.

The average composition of the diet has been considered in terms of seven different classes of foods. Table II shows the range and mean for the consumption of each class of food, per day, per consumption unit (oz.), for each group.

The analyses of the diets, as summarized in Table I, show that they are on the whole adequate as regards total calories. Aykroyd (1937) has discussed at some length the question of suitable dietary standards applicable in India. On account of the warmer climate, somewhat lower basal metabolism, lower physical activity and the generally smaller build of the people, standards commonly accepted in Europe are probably too high to be adopted in this country. Aykroyd and Krishnan (1937) consider about 2,500 calories per day as representing the minimum energy requirement of a south Indian peasant. For these families the average calorie intake per day per consumption unit worked out to be 2,808, varying between 1,924 and 3,914 in the various groups. Sixteen families out of a total of 51 were found to be at a level lower than 2,500. The average intake for these was in the neighbourhood of 2,000 to 2,300. Since the calorie requirement must be regarded in the light of the physical work done by the individual the significance of these low figures may not be stressed. The percentage of calories derived from different constituents of the food are also shown in this table and it may be appreciated that cereals furnish the greater bulk of the total energy requirement.

TABLE I.

Group.	Number.	PARTICULARS OF FAMILY.		MAJOR CONSTITUENTS (g.).			FROM ANIMAL SOURCE (g.).		MINERALS (g.).			CALORIES.				Totals.
		Heads.	Consumption units.	Total protein.	Total fat.	Total carbohydrate.	Protein.	Fat.	Total ash.	Calcium.	Phosphorus.	Percentage protein.	Percentage fat.	Percentage carbohydrates.	Percentage cereals.	
I	1	0	4.6	70	65	343	4.5	55.0	8.21	0.64	1.62	12.5	26.3	61.2	71.0	2,297
	2	10	6.1	67	28	374	0	17.0	8.59	0.50	1.50	12.2	12.7	75.1	81.2	2,047
	3	4	3.3	107	64	652	9.3	41.0	13.01	0.91	2.39	11.8	16.1	72.1	68.8	3,703
	4	3	2.2	97	90	531	10.2	77.7	13.27	0.99	2.28	11.7	24.5	63.8	69.7	3,412
	5	5	4.4	85	51	433	9.0	40.0	11.25	0.88	1.95	13.2	18.25	68.3	74.9	2,597
	6	3	2.8	96	116	516	27.4	104.6	14.32	1.35	2.26	11.0	30.0	59.0	41.7	3,587
	7	6	5.0	81	52	414	12.2	42.0	11.17	0.95	1.89	13.2	19.3	67.5	68.2	2,513
	8	4	2.8	83	29	433	6.0	17.6	11.05	0.82	1.87	14.3	18.7	74.4	78.0	2,385
	9	9	6.2	76	47	388	0.6	38.0	10.27	0.60	1.77	13.3	13.3	68.0	80.3	2,340
	10	2	1.8	106	20	506	1.7	5.7	14.40	1.01	2.45	13.3	19.3	67.4	70.3	3,235
	11	1	1.0	99	65	617	0	24.0	14.12	0.70	2.10	11.5	17.2	71.3	73.4	3,543
	12	3	2.1	73	13	366	1.4	0.3	9.35	0.57	1.70	15.6	6.4	78.0	89.5	1,924
	13	3	3	83	81	399	17.4	76.4	11.70	0.90	1.93	12.7	27.8	59.6	60.4	2,705
	14	1	1.0	119	91	508	0	74.6	16.18	0.82	2.79	14.3	24.8	61.0	79.5	3,417
	15	6	4.3	80	36	407	11.3	26.1	11.10	1.17	1.86	14.1	14.3	71.5	74.5	2,334
	16	6	5.2	120	108	498	36.0	95.5	17.82	1.87	2.54	13.9	28.3	57.8	60.7	3,537
Total	72		55.6 Mean	90.1	59.0	461.5	9.2	45.9	12.20	0.92	2.50	13.0	29.9	67.2	71.4	2,786

TABLE II.

Group.	Rice and atta. (Oz.)	Dals. (Oz.)	Fruits and raw vege- tables. (Oz.)	Vegetables. (Oz.)	Meat, fish, and eggs. (Oz.)	Milk and curd. (Oz.)	Butter and ghee. (Oz.)
I	11.4 to 24.8	0 to 7.2	0 to 8.0	1.8 to 8.5	0	0 to 36.5	0.1 to 2.8
Mean ..	17.4	1.9	2.5	4.6	0	8.7	1.2
II	12.2 to 28.2	0 to 2.6	0 to 4.4	1.9 to 19.0	0 to 4.0	0 to 21.6	0 to 4.0
Mean ..	19.8	0.96	1.6	5.1	1.5	7.8	1.3
III	15.2 to 20.6	0.6 to 5.1	0 to 4.6	0.6 to 6.2	0 to 0.4	1.3 to 23.6	0 to 2.2
Mean ..	17.8	1.64	1.04	4.16	0.05	12.3	1.45
IV	15.2 to 29.4	0.6 to 5.6	0	0.8 to 8.0	0 to 8.8	0 to 8.0	0 to 1.3
Mean ..	22.4	2.7	0	3.32	1.7	2.4	0.4
M E A N FOR ALL GROUPS.	19.4	1.8	1.3	4.3	0.82	7.8	1.09

The average intake of total protein is 98 g. per day per unit when all the families are taken together and varies from 90 g. to 109 g. in the various groups. It would appear obviously to be quite adequate. However, it may be pointed out that a very large proportion of this protein is derived from cereal grains (Table III):—

TABLE III.

Group.	Total protein intake. (g.)	Protein derived from cereal. (g.)	Percentage of protein from cereal.
I	90.1	67.8	75.2
II	97.3	77.2	79.3
III	95.1	69.4	73.0
IV	109.6	87.4	79.8

The proportion of animal protein in all the groups is very low, the intake being 9.2 g. to 18.8 g. This is due to the vegetarian character of the diet, the consumption of milk being low.

The fat intake is 59 g. to 64 g. in the first three groups and over 70 per cent of this is derived from animal source. Group IV families which are at a lower economic level have a fat intake of only 38 g. of which only one-third is from animal source.

The diets have been analysed only for two minerals—calcium and phosphorus. The calcium content of the diets in the various groups varies from 0.76 g. to 1.02 g., while the phosphorus content from 2.20 g. to 2.36 g. This is quite satisfactory according to the common standards. The fact that these diets are mainly cereal must be taken into consideration. The phosphorus of the cereal grains is present largely in the form of phytin, and the phosphorus of the phytin is only sparingly available to the organism. The availability of the phosphorus of the Indian cereals needs serious consideration*.

The results of Table II throw further light on the character of the diet in this area. The average composition of the diet for all the families taken together is as follows :—

	Oz.
Atta	17.8
Rice	1.6
Dals	1.8
Fruits and raw vegetables	1.3
Vegetables	4.3
Meat, fish, and eggs	0.82
Milk and curd	7.8
Butter or ghee	1.09

Wheat is the main cereal, rice being taken only on a few occasions and in small quantities. The intake of dal varies from 0.96 oz. to 2.7 oz. per day in different groups, group IV which is at the lowest economic level consuming the highest quantity and group II the smallest. Group I stands the second highest.

The intake of fruit or raw vegetables is very small. Twenty-five out of the 51 families are taking none at all. It is completely absent from the diet of group IV families. Group I families which have the highest intake consume 2.5 oz. per day, while groups II and III 1.0 oz. to 1.6 oz. The intake of cooked vegetables varies from 3.3 oz. to 5.1 oz., group IV representing the lowest level.

The common vegetables consumed were potatoes, brinjals, turnips, pumpkin, gourd, cauliflower, etc., the green leafy vegetables being almost absent. These vegetables are known to be poor in vitamin C. It is possible that this fact when added to the possibly adverse Ca : P ratio of the food as pointed out above may have some bearing on the relatively high incidence of dental defects. Wilson, Ahmad and Mitra (1937) reported an incidence of 35.3 per cent dental caries, and 30.3 per cent other dental defects in the children of this area.

* The average consumption of wheat atta by the families is 19.4 oz. This quantity contains about 1.68 g. phosphorus. If only about 25 per cent of this phosphorus is available, the average amount of 'available' phosphorus furnished by these diets falls to a figure as low as 1.07 g.

Meat, fish, and eggs are completely absent from the diet of families in group I, in group III only negligible quantities are consumed, while in group IV only three families out of 8 are taking meat. One of these taking high quantities of meat is rather an exception. The man keeps two dogs and every morning goes out into the fields hunting rabbits. Group II is the only group showing a consumption of any appreciable quantities of meat, fish, or eggs, though even in this group the average intake was only 1.5 oz. per day. The dietary habits of Hindus (group I) are well known to be vegetarian.

The quantity of milk and curd in some form or other in the diet varied from 7.8 oz. to 12.3 oz. per day in the first three groups, group III taking the highest and group II the lowest amount. These quantities could be considered adequate if other sources of animal protein and fat were also present in the diet. The diets, however, are essentially vegetarian with the exception of group II and it has already been pointed out that the diets are low in animal protein. These quantities of milk are inadequate for the needs of children and nursing and pregnant mothers. At the time of the examination of 1,250 school children of this area information was obtained regarding the number of children drinking milk regularly and it was found that only 351 children took milk daily. The quantity of milk taken by the great majority of these children was not more than 8 ounces per day per head. This quantity is probably inadequate for growing children.

It may be of interest to add a few general observations with regard to the supply and intake of milk. At the economic level we are considering only a few families buy milk in any appreciable quantities for drinking. When milk has to be purchased it will be a small quantity, just enough to make tea or occasionally a pudding. Curd may be purchased if no vegetable or dal has been cooked to eat with the bread as a meal. Thus the milk intake of such families as have to buy it is very small. If, however, economic and other circumstances permit, the tendency for families is to own a cow or a buffalo. In such cases the milk supply is plentiful, but the whole of the daily supply is soured and converted into lassi (buttermilk) and ghee. Lassi is separated and diluted largely. It is an excellent beverage in the warm and dry spring and summer days and there is no question of its high nutritive value. It is taken by the adults in fairly large quantities, but children take only a small quantity of it. Though a valued drink, lassi is regarded as a by-product in the production of ghee and is very often distributed free to neighbours when in excess of the requirements of the family. Ghee is utilized for the cooking of food and often saved up and sold.

Even in such families where the apparent supply of milk is plentiful the allowance to children is not very generous, the average being about 8 oz. per day. The mother is anxious to save milk to get the maximum quantity of ghee. The cow is an economic asset. All 351 children mentioned above as milk consumers belonged to families owning a cow or buffalo.

The intake of ghee was found to be 1.2 oz. to 1.45 oz. per day per consumption unit in the families of groups I, II, and III. In the case of group IV the consumption was only 0.4 oz. It is a general observation that with the lowering of the economic level the intake of fat suffers the first cut.

SUMMARY AND CONCLUSIONS.

The diets of fifty-one families of Ferozepore, Punjab, belonging to different communities and at two different economic levels have been investigated. It is found that the quantity of food as regards energy requirement is adequate. The total protein intake is satisfactory though 73 to 80 per cent of protein is furnished by cereal. The quantity of animal protein in the diet is low and possibly inadequate for growth, pregnancy, and lactation.

The quantity of fat in the diets appears to be adequate but it is interesting to note that families at a higher economic level have a fat consumption of about 61 g. per day over 70 per cent of which is butter fat, while families at a lower economic level have an intake of 38 g. of which only 33 per cent is from animal sources.

The calcium and phosphorus content of the diets may be adequate but since about 73 per cent of total phosphorus is furnished by wheat atta in which it is present in a poorly available form (phytin) the question deserves further investigation.

Wheat atta constitutes the bulk of these diets. The intake of fruit and raw vegetables is very low, 50 per cent of the families getting none at all. The intake of cooked vegetables is also on the low side, the green leafy vegetables being almost absent.

The diet of large groups of the community is vegetarian. Milk and milk products are the only source of animal protein and since their supply in the diet is not very generous the diets are on the whole low in animal protein, particularly for the needs of children, pregnancy, and lactation.

The supply of animal fats in the diet seems to be satisfactory.

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PYROPHOSPHATE IN THE DETERMINATION OF VITAMIN-C CONTENT OF PLANT AND ANIMAL TISSUES.

BY

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IN a previous paper (Giri, 1937) some observations were recorded concerning the protective action of pyrophosphate against the oxidation of vitamin C, and it was suggested that pyrophosphate could be used as a reagent for the extraction of vitamin C from plant and animal tissues, with a view to preventing the oxidation of the vitamin during extraction.

Several methods have been proposed for the extraction and determination of vitamin C and some of them have the common feature that they involve the stabilization of the vitamin during extraction. It is well known that traces of copper and iron, which occur as impurities in the ordinary routine analysis of vitamin-C content of foodstuffs, catalyse the oxidation of the vitamin. Further, both the water and the reagents used may contain copper in such concentrations as to cause a rapid oxidation of the vitamin during extraction, thus resulting in lower titration values.

The inhibiting action of various substances on the catalytic oxidation of vitamin C has been studied by several workers, with a view to minimizing errors in the routine methods for the determination of the vitamin. Among the various reagents, particular attention may be drawn to the metaphosphoric-acid method devised by Fujita and Iwatake (1935), and subsequently developed by other workers (Musulin and King, 1936; Levy, 1936; Hinsberg, 1937). The use of such reagents in the chemical examination of vitamin-C content of plant and animal tissues has been discussed previously (Giri, *loc. cit.*) and the merits and demerits of the various known reagents for the stabilization of vitamin C were considered.

The present inquiry was undertaken to investigate the use of pyrophosphate as a stabilizing agent in the extraction and determination of vitamin-C content of plant and animal tissues.

EXPERIMENTAL.

The materials used in the present study were obtained fresh from the market, and immediately used for extraction of the vitamin. The edible portion of the material was always taken for analysis, and the results are expressed in mg. of vitamin C present in 100 grammes of the fresh edible portion of the material.

Extraction.—The material was cut into small pieces, and divided into two equal portions each weighing 10 g. To one portion 30 ml. of 20 per cent trichloroacetic acid were added, and this was followed by 5 g. of pure, acid-washed sand. The material was ground well in a glass-mortar, and immediately centrifuged. The supernatant was decanted and the residue was again extracted with 20 ml. of 20 per cent trichloroacetic acid, and again centrifuged. The two extracts were mixed together and diluted to 200 ml. so that the final extract contained 5 per cent trichloroacetic acid. The extract was then filtered and titrated without delay. The other 10 g. portion of the material was extracted with a mixture of 20 per cent trichloroacetic acid and 2 per cent pyrophosphate, and treated similarly.

Titration.—The titration and standardization of vitamin-C solution and the preparation of sodium pyrophosphate used in the present investigation followed the lines indicated in the previous paper (Giri, *loc. cit.*). The results are presented in Table I:—

TABLE I.
Vitamin-C content of foodstuffs.
Vitamin C in mg. per 100 g. of fresh material.

Number.	Name of material.	Botanical name.	EXTRACTION.	
			Trichloroacetic acid alone.	Trichloroacetic acid plus pyrophosphate.
	1. <i>Plant materials.</i> —			
1	Apple	<i>Pyrus malus</i>	4.0	7.0
2	Betel leaves	<i>Piper betle</i>	11.0	12.0
	„ another variety, tender leaves.	„	10.0	12.0
3	Brinjal	<i>Solanum melongena</i>	5.0	7.0

TABLE I—contd.

Number.	Name of material.	Botanical name.	EXTRACTION.	
			Trichloroacetic acid alone.	Trichloroacetic acid <i>plus</i> pyrophosphate.
	1. <i>Plant materials</i> —concl'd.			
4	Cabbage	<i>Brassica oleracea capitata</i>	51·0	69·0
	„ (after keeping the extract at room temperature for 3 hours).	„	42·0	59·0
5	Carrots	<i>Daucus carota</i>	6·0	8·0
6	Chillies (green)	<i>Capsicum annum</i>	47·0	67·0
7	Cluster beans	<i>Cyamopsis psoralioides</i>	33·0	36·0
8	Coriander leaves	<i>Coriandrum sativum</i>	58·0	76·0
9	Drumstick leaves	<i>Moringa oleifera</i>	204·0	230·0
	„ (after keeping the extract at room temperature for 24 hours).	„	94·0	177·0
10	Indian gooseberry (<i>Amla</i>)	<i>Embolica officinalis</i>	639·0	729·0
	„ (pulp dried at room temperature and powdered).	„	..	2,547·0
11	Orange	<i>Citrus aurantium</i>	25·0	33·0
12	Papaya (ripe)	<i>Carica papaya</i>	40·0	49·0
	„ (after keeping the extract at room temperature for 24 hours).	„	19·0	43·0
	Papaya (unripe)	„	16·0	20·0
13	Plantain (ripe)	<i>Musa paradisiaca</i>	7·0	9·0
	„ (unripe) (different variety).	„	12·0	15·0
14	Potato	<i>Solanum tuberosum</i>	6·0	8·0
15	Pumpkin (sweet)	<i>Cucurbita maxima</i>	5·0	6·0
16	Radish	<i>Raphanus sativus</i>	26·0	28·0
17	Sweet potato	<i>Ipomœa batatas</i>	3·0	4·0
18	Tomato	<i>Lycopersicum esculantum</i>	13·0	22·0

TABLE I—*concl'd.*

Number.	Name of material.	Botanical name.	EXTRACTION.	
			Trichloroacetic acid alone.	Trichloroacetic acid <i>plus</i> pyrophosphate.
2. <i>Animal tissues.</i> —				
	Sheep—			
1	Liver	22·0	26·0
	„ (after keeping the extract at room temperature for 24 hours).	11·0	20·0
2	Kidney	14·0	18·0
	„ (after keeping the extract at room temperature for 24 hours).	11·0	16·0
3	Brain	19·0	23·0
	„ (after keeping the extract at room temperature for 24 hours).	12·0	20·0

Preparation of standard solution of vitamin C.—For the preparation of standard vitamin-C solutions, B. D. H. ascorbic acid was used. The vitamin was dissolved in 5 per cent trichloroacetic acid containing 0·5 g. of pyrophosphate in 100 ml. of the solution. It was found that the vitamin was quite stable in presence of pyrophosphate. The following figures (Table II) show the usefulness of pyrophosphate as a reagent for the preparation of standard solutions of the vitamin :—

TABLE II.

Stabilization of vitamin C by pyrophosphate and trichloroacetic-acid mixture.

Time in hours at room temperature.	VITAMIN C IN MG. IN 100 ML. OF SOLUTION.	
	Without pyro-phosphate.	With pyro-phosphate.
0	40·0	40·0
2	30·0	40·0
24	4·35	38·4

DISCUSSION.

The results of the present investigation have confirmed the previous finding that pyrophosphate has a definite protective action against the oxidation of vitamin C (Giri, *loc. cit.*). The higher values obtained for vitamin C when pyrophosphate-trichloroacetic-acid mixture was used for extraction of the vitamin suggests that pyrophosphate protects the vitamin against oxidation during extraction. In the absence of the stabilizing agent the oxidation of vitamin C occurs during the process of extraction, and therefore the values obtained for the vitamin-C content of plant and animal materials are found to be low. In the light of these results, the addition of pyrophosphate to trichloroacetic acid used for extraction of vitamin C from plant and animal tissues is recommended, since it inhibits the oxidation of the vitamin. Further, by using such stabilizing agents, the necessity for hydrogen-sulphide treatment, which is generally employed for reducing the reversibly oxidized vitamin C, disappears. It has been found that when hydrogen-sulphide reduction is used, the time of treatment should not exceed 30 minutes to avoid reduction of substances other than vitamin C (Mack and Tressler, 1937). The interference of such substances arising as a result of hydrogen-sulphide treatment can be avoided or minimized by the use of a stabilizing reagent such as pyrophosphate.

The figures in Table I show that in general the green leaves contain much vitamin C. The values obtained for the vitamin-C content of some of the foodstuffs investigated, have been found to vary from those reported by Ranganathan (1935). Particularly in the case of leafy vegetables, the difference in the two sets of values has been found to be about 50 to 100 per cent. The vitamin content of papaya has been found to increase as the fruit ripens.

Vitamin-C content of Indian gooseberries (Amla).—One of the significant findings of the present investigation is the occurrence of vitamin C in very high concentration in Indian gooseberries. That the berries are the rich sources of the vitamin has already been pointed out by Damodaran and Srinivasan (1935). They have shown that 1 g. of fresh material contains about 4.13 mg. vitamin C. Our results show that the edible fleshy portion contains as much as 7.29 mg. per 1 g. of fresh material. Even after mercuric acetate treatment, which removes reducing materials other than vitamin C, Damodaran and Srinivasan (*loc. cit.*) found that about 3.65 mg. of vitamin C are present in 1 g. of fresh material. It can be seen from the results in Table I that the powder obtained by drying the pulp of the berries at room temperature contains about 25 mg. of vitamin C in 1 g. of the dry powder.

These results are of importance in view of the fact that the berries are being extensively used in Ayurvedic preparations. One such preparation, which contains mainly the powdered pulp of the berries, is *Chyavanaprash*. This is reputed to cure cough, asthma, consumption, and other lung diseases, and also senile decay. Since the berries contain about 7 mg. of vitamin C per gramme of fresh pulp, the whole normal requirement of vitamin C can readily be met by eating one or two berries a day. This quantity, if taken every day, should be capable of saturating human beings with vitamin C.

Further work on the isolation and identification of vitamin C in pure state from the berries is in progress.

SUMMARY.

The application of a pyrophosphate-trichloroacetic-acid mixture in the determination of vitamin-C analysis of plant materials and animal tissues, and in the preparation of stable aqueous solutions of vitamin C, is described.

Among the plant materials analysed, Indian gooseberries are the richest source of vitamin C. Further work is in progress on the isolation of the vitamin from gooseberries in pure form.

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VITAMIN C AND PEPTIC ULCER.

BY

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VITAMIN deficiency was considered by Bradfield (1927), McCarrison, and others as one of the probable causes of the increased incidence of peptic ulcer in South India. Vitamin-C deficiency has also been suggested as a probable ætiological agent in the causation of peptic ulcer. Experimental evidence in support of this thesis is also forthcoming. Roseno (quoted by Smith and McKonkey, 1933) observed that the occasional occurrence of peptic ulcer in the cow and the sheep was due to their feeding on dry pasture, devoid of vitamin C. Smith and McKonkey (*loc. cit.*), by feeding guinea-pigs on diet deficient in vitamin C, observed that 26 per cent of the animals developed acute hæmorrhagic lesions of the stomach and duodenum and that vitamin-C deficiency aids also in the chronicity of an artificially produced ulcer. Observations which might be given a similar interpretation have also been made previously by McCarrison (1921).

On the basis of these experimental findings it may be concluded that vitamin-C deficiency may predispose to ulcer formation in the stomach and duodenum in animals. It was the object of the present investigation to study whether such a deficiency is the cause of the common occurrence of peptic ulcer in South India.

Ray *et al.* (1935) have developed a microchemical technique by which partial avitaminosis C can be made out by repeated examination of urine for ascorbic acid excretion. On the basis of this technique Harris *et al.* (1935) devised a test for demonstrating a relative deficiency of vitamin C in the body. If a patient having adequate quantities of vitamin C in the diet is made to ingest massive quantities of ascorbic acid, a large percentage of the extra ascorbic acid is excreted in the urine; whereas in a person with an inadequate supply of vitamin C a similar

massive dose of ascorbic acid leads to the excretion of only a small quantity, the remaining amount being utilized to saturate the body. On this basis they have evolved a method, by which the degree of vitamin-C sub-nutrition can be estimated from the amount of vitamin excreted, following the ingestion of a constant known daily supply of the vitamin by the patient. Archer and Graham (1936), however, suggest that the percentage output is much more valuable evidence that a patient has incipient scurvy than either the amount excreted after a test dose or the amount of ascorbic acid to be given to bring the excretion to a certain level. The condition of vitamin-C nutrition in peptic ulcer patients has been studied by the above method. The technique is based on the capacity of ascorbic acid to decolorize in an acid medium, dichlorophenol-indophenol indicator. For brevity only the important experimental details are given below :—

1. The urine is collected in dark bottles and over glacial acetic acid. The amount of urine taken is such that the concentration of the acid in urine before examination is not less than 10 per cent.
2. The urine is examined as soon as possible after it is passed. In the present investigation the urine was examined twice in the day; once in the early morning for the night urine, and once in the evening for the day urine. It is, however, stated that urine may be kept with comparatively little loss (by oxidation) for about 12 to 15 hours, with the addition of 5 per cent acetic acid (Harris *et al.*, 1935).
3. The actual titration should not take more than two minutes as substances other than ascorbic acid are supposed to reduce this indicator slowly.
4. The strength of the stock dye solution was 0.1 per cent and the titration was done in such a way that 1 c.c. to 2 c.c. urine required 0.025 c.c. to 0.05 c.c. indicator. In the case of more concentrated urines the urine was diluted proportionally. (In the present investigation the dye solution was prepared daily and standardized daily, as the results were found to show a greater experimental error when titrations were done with old solutions.)
5. The daily test dose was given in the proportion of 5 mg. of ascorbic acid for every pound of body-weight.

Blood.—The principle of ascorbic-acid estimation in blood involves the removal of interfering substances (Emmerie, 1934) from the deprotenized blood, and titrating the reducible ascorbic acid (Tillman *et al.*, 1932, quoted by Emmerie. *loc. cit.*) in an acid solution (Birch *et al.*, 1933) with standard 2 : 6 dichlorophenol-indophenol solution. The method of Emmerie and Marie van Ekelén (1934) was followed.

Table I represents the ascorbic-acid content of blood in fifteen cases of peptic ulcer (verified by biochemical and radiological investigations and/or operation).

TABLE I.

Serial number.	Name.	Ascorbic acid (mg. per 100 c.c. of blood).	Serial number.	Name.	Ascorbic acid (mg. per 100 c.c. of blood).
1	N.	0.4180	9	K. R. M.	0.5044
2	A. T.	0.4118	10	Ch. S.	1.753
3	A. S.	0.7875	11	S.	1.647
4	B. R.	0.5094	12	B. V. R.	1.545
5	S. M. S.	0.7278	13	P. P. U.	0.4054
6	K.	1.1427	14	G. K.	0.5792
7	E. N.	1.920	15	R. S.	0.4856
8	P. T.	0.6200			

COMMENT.

The vitamin-C content of the blood in peptic ulcer patients, as shown in Table I, shows a variation from 0.4054 mg. to 1.920 mg. with an average of 0.8971 mg. That these values are nearly identical with values in healthy controls (as reported by various authors) is seen by comparison with Table II :—

TABLE II.

	Ascorbic acid (mg. per 100 c.c. of blood).
1. Emmerie and Marie van Ekelen (<i>loc. cit.</i>)	0.4165 to 1.04125
2. Narayana Menon (1935)	0.714 to 1.275
3. Mirsky <i>et al.</i> (1935) (quoted by Stephens and Hardley, 1936).	1.11 to 2.88
4. Deggeller (1936)	0.5 to 1.3
5. Marie van Ekelen (1936)	0.4 to 1.3

One is thus justified in concluding that there is no gross vitamin-C deficiency in patients suffering from gastric or duodenal ulcers.

Nine patients with duodenal ulcer have been examined by the test dose method and the following are the results in a tabular form. All the observations are made on patients soon after their admission so that the differences in their ascorbic-acid saturation values owing to the hospital diet are minimal.

lack of vitamin C and vitamin P. According to his conception, it is a group of substances—flavones (tentatively termed vitamin P) that are responsible for the favourable effect on the resistance and permeability of the capillary wall in pathological conditions. Hence it may be that though deficiency of vitamin C is present there is an adequate amount of vitamin P and this prevents the development of scurvy. The rarity of reports of genuine cases of scurvy in South India lends evidence to the same. Moreover, it has been observed (Narasimha Rao, 1938) in a dietetic survey that the diet of the labourer, in whom peptic ulcer is found most commonly to occur, is not markedly deficient in vitamin C. It seems, therefore, that low vitamin-C reserves found in peptic ulcer patients have probably no relation to the chronicity of the ulcer.

SUMMARY.

1. The ascorbic-acid content of the blood in patients suffering from peptic ulcer has been estimated and found to be within normal limits. Patients with peptic ulcer as well as healthy controls show incomplete saturation with vitamin C as judged by Harris and Ray's test dose method.

2. Vitamin-C deficiency is probably not an ætiological factor in the formation of peptic ulcer.

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NUTRITIONAL INVESTIGATION OF SOME SPECIES OF BENGAL FISH.

Part I.

BIOLOGICAL VALUE OF THE PROTEINS OF RUHEE (*LABEO
ROHITA*) AND HILSA (*CLUPEA ILISA*) BY THE NITROGEN-
BALANCE AND GROWTH METHODS, AND SUPPLE-
MENTARY EFFECT OF FISH ON
PULSE PROTEINS.

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FISH is an important article of foodstuff in Bengal and is taken almost daily. Fish supplies animal protein an adequate provision of which in Indian dietaries is very important. The liver and body oils of some varieties of Indian fish might prove to be very rich sources of vitamins A and D. The different varieties of fish are not very probably of the same nutritional value and as yet no systematic investigation of the Bengal fish has been undertaken from this standpoint.

Our investigations would include a systematic study of the protein content and its biological value, vitamins A and D and sterols of the body and liver oils, and also of the mineral constituents of the different varieties of Bengal fish.

The present paper deals with the determination of the quality of the proteins of the two very common varieties of fish in Bengal, the *Labeo rohita* (ruhee) and the *Clupea ilisa* (hilsa) and also of the effect of mode of preparation of the meal on the quality of the proteins.

Schneider (1932) made the nitrogen balance studies with various fish meals of America and found the biological value of white fish meal to be 84. Later on Maynard and Tunison (1934) re-investigated the same with menhaden fish meal and supported Schneider's result. Wilder, Bethke and Record (1934) using nitrogen balance in rats determined the biological value of haddock fish meal after varying heat treatment for the preparation of the meal.

In this investigation the biological value of the fish proteins has been determined at different levels of protein intake (5, 10, and 15 per cent protein in the diet) both by the balance-sheet method and by the growth of young rats. The technique followed in this case was the same as in the previous investigations in this laboratory (Basu *et al.*, 1936a and b; Basu and Basak, 1937; and Basu, Nath and Mukherjee, 1937).

COMPOSITION OF FISH AND THE PREPARATION OF THE FISH MEAL.

The head, tail, and bones of the fish were rejected. The fish was cut into thin slices and put into the steam oven. When the slices were partially dry, they were chopped in a small chopper and finally dried in a steam oven. The nitrogen content of the meal was determined. In the case of hilsa fish a part of the oil was pressed out.

The sun-dried meal was prepared by drying in the sun after partially drying in the steam oven.

The composition of the whole fish and the meals are indicated in Table I:—

TABLE I.

Variety.	Crude protein, per cent.	Moisture, per cent.	Ether extract, per cent.	Ash constituents, per cent.
Ruhee fish (whole) ..	23.28	..	4.2	..
Hilsa (whole)	19.67	..	20.59	..
Ruhee meal (steam-dried) ..	72.24	5.28	3.87	12.40
Ruhee „ (sun-dried) ..	76.56	6.92	2.24	8.38
Hilsa „ (steam-dried) ..	60.0	4.21	16.23	6.41
Hilsa „ (sun-dried) ..	64.28	6.49	12.34	4.59

COMPOSITION OF THE DIETS.

The composition of the diets is indicated in Table II:—

TABLE II.

Composition of the diets.

Constituents.	Nitrogen-free.	PROTEIN IN DIET.							
		STEAM-DRIED HILSA.			STEAM-DRIED RUHEE.			SUN-DRIED	
		5 per cent.	10 per cent.	15 per cent.	5 per cent.	10 per cent.	15 per cent.	RUHEE.	HILSA.
								10·2 per cent.	10 per cent.
Hilsa (steam)	50	100	150
Ruhee (steam)	42	84	126
Ruhee (sun)	78	..
Hilsa (sun)	94
Ghee (butter-fat)	72	64	56	48	72	72	72	72	60
Chopped sugar ..	54	54	54	54	54	54	54	54	54
Salt mixture ..	30	24	24	24	24	24	24	24	24
Cod-liver oil ..	12	12	12	12	12	12	12	12	12
Calcium carbonate	6	6	6	6	6	6	6	6	6
Starch ..	426	390	348	306	390	348	306	354	350

I. *Experiments by the balance-sheet method.*—Typical data regarding the metabolism experiments and calculations of the biological values for 10 per cent protein level of hilsa and ruhee proteins in the diet are indicated in Table III. The results of experiments at different concentrations of protein in the diet are summarized in Table IV.

Experiments with nitrogen-free diet were done at the beginning and also at the end of each series. In calculating the biological values, mean values for the endogenous urinary nitrogen and for the metabolic nitrogen per gramme of food intake were taken from the two experiments with nitrogen-free diet.

II. *Experiments by the growth method.*—The quality of the two fish proteins has also been determined by the growth induced in young rats per gramme of protein intake over a period of four and of eight weeks. Experiments were carried out at 5, 10, and 15 per cent protein concentrations in the diets, the composition of which was the same as given in Table II. The growths obtained and the biological values $\left[= \frac{\text{gain in weight (g.)}}{\text{protein intake (g.)}} \right]$ at 10 per cent protein level are indicated in Table V, and the results at different protein concentrations are summarized in Table VI. The technique employed was the same as used in the previous investigations from this laboratory (Basu, Nath and Ghani, 1936a and b; Basu, Nath and Mukherjee, *loc. cit.*; Basu and Basak, *loc. cit.*).

III. *Effect of sun-drying.*—As sun-dried fish is consumed in some parts of Bengal, the effect of drying the fish in the sun instead of drying it in the steam on the quality of the proteins has been investigated both by balance-sheet and growth methods.

TABLE III.
Balance-sheet experiments with proteins of hilsa and ruhee.
(Figures of intake and excretion represent daily averages.)

Rat number.	Initial weight, g.	Final weight, g.	Food intake, g.	Nitrogen intake, mg.	Faecal nitrogen, mg.	Metabolic nitrogen, mg.	Food nitrogen in faeces, mg.	Absorbed food nitrogen, mg.	Total urinary nitrogen, mg.	Endogenous urinary nitrogen, mg.	Food nitrogen in urine, mg.	Food nitrogen utilized, mg.	Biological value.	Mean biological value.
<i>Experiment with nitrogen-free ration.</i>														
316	90	87.5	4.2	..	7.98	1.91 a	24.28
317	131	127.0	6.8	..	14.41	2.12 a	26.42
294	150	147.5	4.2	..	7.9	1.9 a	28.4
295	177	172.5	6.3	..	12.47	1.97 a	31.84
296	200	193.0	7.0	..	14.02	2.0 a	43.65
297	250	243.0	9.0	..	20.74	2.3 a	51.32
<i>Experiment with 10 per cent hilsa-fish (steam-dried) protein.</i>														
316	94	96.5	4.1	65.6	25.66	8.61	17.05	48.55	44.03	24.04	19.09	29.46	61	..
317	130	132.0	6.1	97.6	27.50	12.41	15.19	82.41	53.93	28.31	25.62	56.79	69	..
294	152	153.5	6.4	102.4	24.36	11.52	12.84	89.56	52.04	30.1	22.54	67.02	74	..
295	180	181.5	6.7	107.2	28.59	12.73	15.86	91.34	59.19	32.28	26.91	64.43	70	69.5 ± 1.34
296	205	206	8.0	128.0	38.66	15.20	23.46	104.54	69.15	40.68	28.47	70.07	75	..
297	260	262	10.1	161.6	46.5	22.22	24.28	137.32	97.84	54.75	43.09	94.23	68	..

a = per gramme of food.

TABLE III—*concd.*
(*Figures of intake and excretion represent daily averages.*)

Rat number.	Initial weight, g.	Final weight, g.	Food intake, g.	Nitrogen intake, mg.	Faecal nitrogen, mg.	Metabolic nitrogen, mg.	Food nitrogen in faeces, mg.	Absorbed food nitrogen, mg.	Total urinary nitrogen, mg.	Endogenous urinary nitrogen, mg.	Food nitrogen in urine, mg.	Food nitrogen utilized, mg.	Biological value.	Mean biological value.
<i>Experiment with 10 per cent ruhee-fish (steam-dried) protein.</i>														
316	91	101.5	7.0	112.0	24.77	14.7	10.07	101.93	45.31	24.94	20.37	81.56	80	78.9 ± 0.95
317	141	143	7.8	124.8	33.72	15.87	17.85	106.95	47.35	28.31	19.04	87.91	82	
294	150	162	8.2	131.2	30.45	14.76	15.69	115.51	56.88	30.1	26.78	88.73	77	
295	187	193	9.0	144.0	25.73	17.10	8.63	135.37	65.70	32.28	33.42	101.95	75	
296	212	216.5	10.4	166.4	35.99	19.76	16.23	150.17	65.45	40.68	24.77	125.40	83	78.9 ± 0.95
297	269	271.5	11.3	180.8	44.24	24.86	19.38	160.42	82.38	54.75	37.63	122.79	76	
<i>Experiment with nitrogen-free ration.</i>														
316	108	102.5	5.0	..	11.5	2.3 a	25.61
317	150	143.5	7.2	..	14.04	1.95 a	30.19	
294	162	153	6.1	..	10.37	1.7 a	31.8	
295	198	190	7.2	..	13.17	1.83 a	32.72	
296	221	212	8.5	..	15.38	1.8 a	37.72	
297	272	284	10.8	..	22.69	2.1 a	58.18	

a = per grammes of food.

TABLE V.

Growth experiments with proteins of ruhee and hilsa.

Rat number.	Sex.	Initial weight, g.	4 WEEKS.				8 WEEKS.					
			Food intake, g.	Protein intake, g.	Gain in weight, g.	B. V. gain in weight, = protein intake.	Mean B. V.	Food intake, g.	Protein intake, g.	Gain in weight, g.	B. V. gain in weight, = protein intake.	Mean B. V.
10 per cent ruhee-fish (steam-dried) protein.												
322	Male ..	48.5	193.4	19.34	38.0	1.96	1.9	353.7	35.37	54.5	1.54	1.55
323	Male ..	40	168.6	16.86	34	2.0		281.8	28.18	46.5	1.65	
324	Female	49.5	160.7	16.07	30	1.86		315.4	31.54	47	1.49	
325	Female	50	158.5	15.85	32	2.0		287.9	28.79	45.5	1.58	
326	Male ..	47	162.4	16.24	31.5	1.93		300	30	48	1.6	
327	Female	40	180	19	31	1.63		331	33.10	48	1.45	
10 per cent hilsa-fish (steam-dried) protein.												
298	Male ..	29	106.2	10.62	17	1.60	1.66	181.2	18.12	24.0	1.32	1.32
299	Female	29.5	80.3	8.03	14	1.74		150.3	15.03	20.5	1.36	
300	Female	30	79.8	7.98	12.5	1.56		162.4	16.24	20	1.23	
301	Female	29	86.2	8.62	14.5	1.68		140.6	14.06	20	1.42	
302	Male ..	32	79.7	7.97	14	1.75		168.4	16.84	21	1.24	
303	Male ..	29	95.4	9.54	15.5	1.62		148.2	14.82	20.5	1.38	

TABLE VI.

Biological value of the proteins of hilsa and ruhee by the growth method.

Material.	Protein in the diet, per cent.	Number of rats.	Period of experiment, weeks.	INCREASE IN WEIGHT, g.		TOTAL FOOD INTAKE (DRY), g.		Protein intake, g. (mean).	B. V. gain in weight. = protein intake.
				Variation.	Mean.	Variation.	Mean.		
Hilsa fish ..	5	6	4	(-1) - 3	1.1	74.2 - 101	88.1	4.4	..
			8	1.5 - 4.5	2.1	132.4 - 179	162.9	8.14	..
	10	6	4	12.5 - 17	14.6	79.7 - 106.2	88	8.8	1.66
			8	20 - 24	21	140.6 - 181.2	158.5	15.85	1.32
	10 (sun-dried)	6	4	25 - 42	36.1	153.7 - 192.6	165	16.5	2.18
			8	45 - 69	56	204 - 373.4	338	33.8	1.65
	15	6	4	20 - 30	26	83.2 - 118	97.7	14.64	1.77
			8	42 - 50	47.4	187.4 - 253.4	212.4	31.86	1.48
	5	6	4	15 - 20	18	196 - 245	214	10.7	1.72
			8	24 - 28.5	26.7	322 - 387	362.6	18.13	1.47
Ruhee fish ..	10	6	4	30 - 38	32.75	159.5 - 193.4	172.2	17.22	1.90
			8	47 - 54.5	48.25	281.8 - 354	311.6	31.16	1.55
	10 (sun-dried)	6	4	50 - 61	53	200 - 253	226	22.6	2.34
			8	80.5 - 112	97	476 - 589	528.3	52.83	1.83
	15	6	4	31 - 59.5	45.5	103.2 - 174	143.4	21.51	2.1
			8	67 - 96	83.2	297.4 - 353	323.6	48.54	1.71

EFFECT OF SEX UPON GROWTH PER GRAMME OF PROTEIN.

The effect of sex of the rat on the biological value is indicated in Table VII:—

TABLE VII.

Effect of sex on the growth.

Type of the diet.	Protein, per cent	Number of rats used.	Average gain per gramme of protein.
Hilsa (steam dried) ..	10	3 Males	1.31
	10	3 Females	1.34
	15	4 Males	1.55
	15	2 Females	1.40
Ruhee (steam dried) ..	5	4 Males	1.47
	5	2 Females	1.46
	10	3 Males	1.59
	10	3 Females	1.50
	15	2 Males	1.8
	15	4 Females	1.67
Ruhee (sun-dried) ..	10	4 Males	1.87
	10	2 Females	1.75
Hilsa (sun-dried) ..	10	3 Males	1.77
	10	3 Females	1.53

It will be seen from Table VII that with one exception—hilsa (steam-dried) at 10 per cent level—the male rats made a greater gain in weight per gramme of protein consumed than the female ones.

DISCUSSION.

Results obtained indicate that the proteins of *Labeo rohita* are more digestible and are also superior to those of the *Clupea ilisa* both for the maintenance of

nitrogen balance as well as for promoting growth in young rats. In this connection it should be remembered that ruhee fish was dried directly, whereas the hilsa was dried after pressing out a certain amount of oil. A fraction of protein might be lost in the process—and this protein might be of higher biological value. With hilsa diets the food intake was lower than with the ruhee diets. It also appears that, for the same fish, sun-dried meal is superior to the steam-dried variety. The protein in the former is more digestible and shows a higher biological value than the protein in the latter. The rats receiving the sun-dried meal retained more protein and made a larger gain in weight than those on steam-dried fish meal. That high temperatures have a detrimental effect upon the quality of fish proteins were previously observed by Schneider (*loc. cit.*), Maynard *et al.* (1932 and 1934), Invaldsen (1929), and by Wilder *et al.* (*loc. cit.*).

In the determination of biological values by the balance-sheet method live weight of rats did not show any appreciable effect on the biological values. Increase in concentration of proteins in the diet had no effect on the digestibility but lowered the biological values. All these results are in conformity with the previous results of Basu *et al.* (*loc. cit.*) from this laboratory.

Growth experiments showed that, with one exception, the male rats made a greater gain in weight per gramme of protein consumed than the female ones. For the same fish as the concentration of protein in the diet increased, the ratio $\frac{\text{gain in weight (g.)}}{\text{protein intake (g.)}}$ increased. Our calculations, however, show that the ratio $\frac{\text{gain in weight}}{\text{protein consumed} - \text{protein requirement for maintenance}}$ is fairly constant and independent of protein concentration in the diet. Protein requirement for maintenance was obtained graphically from protein-intake and gain in body-weight curves. Our previous results (Basu *et al.*, *loc. cit.*) are thus supported by the present observations.

SUPPLEMENTARY RELATION BETWEEN *Labeo rohita* AND PULSE PROTEINS.

In practical dietetics in Bengal fish, pulses, and rice are taken together and hence the biological values of the proteins of mixed feed are of importance. In previous communications (Basu and Basak, *loc. cit.*) from this laboratory a marked supplementary relation between rice and pulse proteins has been reported, while no supplementary relation could be found between the proteins of green gram and lentil (Basu, Nath and Ghani, 1936*a* and *b*). Recently, Swaminathan (1937) at Coonoor has done some work on the biological value of proteins of mixed feeds containing cereals and pulses. It would be useful to find out if there is any supplementary relation between fish (*Labeo rohita*) and pulse proteins. For this purpose the pulses, lentil, and *Lathyrus sativa* with very low biological values (Basu, Nath and Ghani, 1936*a*; and Basu, Nath and Mukherjee, *loc. cit.*) were selected for investigation by the balance-sheet method, while the supplementary effect of the ruhee fish on the proteins of green gram, lentil, field-pea, and *Lathyrus sativa* has been investigated by the growth method.

Results obtained by the balance-sheet method are summarized in Table VIII. The biological values obtained by the growth method are summarized in Table IX:—

TABLE VIII.

Supplementary relation by the balance-sheet method.

Mixed diet.	Observed biological value.	Calculated biological value.
Lentil protein 2.5 per cent and ruhee 2.5 per cent ..	72	68.5
<i>Lathyrus sativa</i> 2.5 per cent and ruhee 2.5 per cent ..	74	67
Lentil 8 per cent and ruhee 2 per cent	51	41.2
<i>Lathyrus sativa</i> 8 per cent and ruhee 2 per cent ..	60	55.6

TABLE IX.

Supplementary relation by the growth method.

Mixed diet.	Observed biological value.	Calculated biological value.
<i>Lathyrus sativa</i> protein 2.5 per cent and ruhee protein 2.5 per cent ..	1.02	0.78
" " " 9 " " " " 1 " ..	0.50	0.19
Green gram 4 per cent and ruhee 1 per cent	0.68	0.29
Field-pea 9 per cent and ruhee 1 per cent	1.35	1.16
Lentil 4 per cent and ruhee 1 per cent	0.44	0.29
" 9 " " " 1 "	1.39	0.69

Table VIII indicates that there is a supplementary relation between ruhee fish and lentil and *Lathyrus sativa* proteins for the maintenance of nitrogen balance

In calculating the biological value for mixed feeds, however, the biological value of fish protein at 2 or 2.5 per cent level has been taken to be the same as at 5 per cent level of protein. But at 2 or 2.5 per cent level of protein intake the biological value of fish proteins might have a higher value, and in that case the supplementary effect of fish protein on pulse proteins is not very appreciable so far as maintenance requirement is concerned.

It has been observed by Basu, Nath and Mukherjee (*loc. cit.*) that khesari (*Lathyrus sativa*) cannot promote growth in young rats even when fed at 10 per cent level. The present investigation shows that diet containing 9 per cent khesari and 1 per cent ruhee proteins produces appreciable growth in young rats. The diet containing equal parts (2.5 per cent each) of ruhee and khesari proteins at 5 per cent level produces a greater gain in weight than the diet containing 9 per cent khesari and 1 per cent ruhee proteins.

Green gram and lentil as observed by Basu, Nath and Ghani (*loc. cit.*) cannot promote growth in young rats when they are fed on diet containing 5 per cent protein. The results in Table IX show that when green gram or lentil is mixed with ruhee fish in the proportion 4 : 1 at 5 per cent level of protein, the rations possess appreciable values for growth promotion.

The addition of 1 per cent ruhee protein to 9 per cent lentil or field-pea protein at 10 per cent level of protein intake enhances the promotion of growth in young rats. Study of Table IX will show that ruhee fish protein possesses only a slight supplementary effect on the field-pea protein. The greatest supplementary effect is found in the case of *Lathyrus sativa*, lentil, and green gram. Even a slight addition of 1 per cent of ruhee protein to the proteins of the above pulses at 10 per cent protein level will supplement the proteins of the above three pulses.

Since the proteins in fish supplement the growth-promoting effect of the pulse proteins, it is highly probable that the pulse proteins will be utilized more efficiently when they are included in the diet containing fish. Although the proteins in *Lathyrus sativa*, green gram, lentil, and field-pea are of relatively poor quality, the deficiencies of their proteins will be made up when they are fed in combination with ruhee or other varieties of fish.

SUMMARY.

1. The biological values of proteins of steam-dried ruhee fish by the balance-sheet method at 5, 10, and 15 per cent levels of protein intake are 82.3, 78.9, and 72.5, respectively. The corresponding values for hilsa-fish proteins are 78, 69.5 and 62.1 per cent. Body-weight of rats has no effect on the biological value.

2. The biological values of proteins of steam-dried ruhee fish by the growth method at 5, 10, and 15 per cent levels of protein intake are 1.47, 1.55, and 1.71, respectively, while the corresponding values for the steam-dried hilsa-fish proteins are 0.25, 1.32, and 1.48.

3. Sun-dried ruhee-fish meal has a higher value than the steam-dried meal both for the maintenance of nitrogen equilibrium and also for promoting growth in young rats. The sun-dried hilsa-fish meal does not possess any statistical

increased value for the maintenance of nitrogen equilibrium but has a higher value for promoting growth in young rats. The digestibility of the sun-dried product is also greater.

4. Appreciable supplementary relation exists between the proteins of ruhee and proteins of the pulses, *Lathyrus sativa* and lentil as observed by the balance-sheet method. Marked supplementary effect of the proteins of the ruhee fish on the proteins of the pulses, lentil, *Lathyrus sativa*, green gram, and field-pea in promoting growth was observed and this is of great practical importance in human dietetics.

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NUTRITIONAL INVESTIGATION OF SOME SPECIES OF BENGAL FISH.

Part II.

EXTRACTION AND CHEMICAL ANALYSIS OF THE PROTEINS OF RUHEE (*LABEO ROHITA*) AND HILSA (*CLUPEA ILISA*).

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IN the previous paper (Basu and De, 1938), it has been shown that the proteins of the ruhee (*Labeo rohita*) fish are superior to those of the hilsa (*Clupea ilisa*) variety as regards their efficiency in maintaining nitrogen equilibrium and in promoting growth. At 10 per cent protein concentration the biological value of ruhee protein is 78, while that of hilsa protein is 69. As regards growth promotion it is found that ruhee fish at 5 per cent level is sufficient to promote growth and the biological value as measured by growth ratio is 1.47, while at the same level the proteins of the hilsa fish fail to cause any growth. At 10 per cent protein level hilsa fish promotes appreciable growth in young rats but the growth is less than with 10 per cent ruhee proteins. Since the experiments were conducted under identical conditions, i.e., diets were similar in composition in all respects, e.g., vitamins (sufficient amounts of vitamins-B complex, A and D being provided) and mineral matter, etc., except proteins, it was thought that this discrepancy between the two varieties might have been due to the difference in the amino-acid make-up of the proteins in hilsa and ruhee.

No work appears to have been done on the chemical analysis of the proteins of Indian fish. Several analyses of fish proteins have been carried out in Japan and Singapore. Sekima and Akiyama (1926) made a study of the proteins of the muscles of some common fishes in the Pacific coast. They only estimated the arginine, histidine, and lysine contents of the muscle-proteins of anchovy, herring, grouper, and tunny. Dunn (1926) in the same year reported on the essential amino-acid contents of the protamine of sardine. Rosedale (1929), by his modified method,

made a series of analysis of the proteins of the most common fish of Singapore market. He carried out the analysis of the di-amino fraction of the proteins of muscle tissues of red snapper, ikan merah (*Lutianus roscus*), horse mackerel, chenchaura (*Carnax rotler*), tunny, carp, etc. He (Rosedale, 1930) also made an analysis of the mono-amino fraction of the proteins of horse mackerel.

Extraction and chemical analysis of the proteins of the *Labeo rohita* and the *Clupea ilisa* form the subject-matter of the present investigation. Besides furnishing knowledge about the Indian fish these investigations reveal the cause of the differences in the nutritive value of the proteins of the two varieties of fish.

The composition of the whole fish and the fish meals is already given in Table I of the previous paper (Basu and De, *loc. cit.*).

PERCENTAGE OF TOTAL NITROGEN EXTRACTABLE BY DIFFERENT SOLVENTS.

The dried fish meal, for the extraction of the proteins, was freed from oil by extraction with ether. The meal was extracted successively with distilled water, 2 per cent salt solution (optimum salt concentration), 70 per cent cold alcohol, 70 per cent boiling alcohol, and 0.2 per cent caustic soda solution. The procedure employed was the same as used by Basu, Nath, Ghani and Mukherjee (1937) and by Basu and Basak (1937) in this laboratory. The results obtained are given in Table I:—

TABLE I.

Percentage of total nitrogen extracted with

	Water.	NaCl, 2 per cent.	Cold alcohol.	Hot alcohol.	Caustic soda, 0.2 per cent.	Total nitrogen extracted, per cent.
Ruhee ..	32.4	5.2	2.4	0.8	55.6	96.4
Hilsa ..	26.8	9.9	2.1	0.5	53.7	93.0

A small percentage of nitrogen is not accounted for and probably represents nitrogenous substances unextractable with these solvents. It is evident that in both cases the highest percentage of nitrogen is obtained in the caustic soda and water extract. Water extract contains appreciable amounts of globulin. So globulin and glutelin were the two proteins investigated.

WATER EXTRACT (GLOBULIN AND ALBUMIN).

About 500 grammes of dried fish meal were extracted with 2½ litres of distilled water for two hours and allowed to settle for about an hour. The practically clear liquid was decanted off and the process of extraction with water repeated twice. The aqueous extract was freed from insoluble particles by centrifuging in a powerful supercentrifuge (36,000 r.p.m.). Thus, the difficulty in filtration encountered by many was easily avoided.

It is interesting to note that a protein was found to separate from the clear extract when the latter was preserved in a refrigerator. The protein coming out of the extract was soluble in water at 40°C. It was purified by repeated precipitation by cooling and by dialysis of the aqueous solution in distilled water. The 'albumin A' was finally washed with absolute alcohol and ether. The amount of protein was, however, too small to be analysed.

The clear solution after removal of albumin A was made half-saturated with ammonium sulphate. Large quantities of precipitate came down. The precipitate was separated by a supercentrifuge and was then dissolved in 2 per cent salt solution and the protein was purified by dialysis first for four days in a current of tap water and then for three days in a current of distilled water. The process of solution and subsequent dialysis was repeated several times. The protein was washed with absolute alcohol and ether and preserved as globulin for analysis.

The clear filtrate after half-saturation was made fully saturated with ammonium sulphate. A precipitate came down which was soluble in water. A protein came down on dialysing the aqueous solution for four days in a current of tap water and for three days in a current of distilled water. The fraction was suspected to be globulin. The clear dialysate was heated on water-bath very slowly and coagulation of the protein occurred at 45°C. The temperature was kept constant and the coagula separated, washed with alcohol and ether and stored as globulin. The amount of albumin A, however, was very small.

Hilsa fish.—A portion of the protein came out of the clear extract on cooling in the refrigerator. It was partially soluble in water at 40°C. The soluble fraction after purification was stored as albumin A. The amount of albumin A of hilsa is very small compared with that of the ruhee.

Globulin in large quantity was found by making the clear extract half-saturated with ammonium sulphate. This was purified in the same way as in the case of ruhee globulin.

The clear filtrate from the globulin fraction, on making fully saturated with ammonium sulphate, gave a precipitate a fraction of which was soluble in water. Globulin was separated from this fraction by the process of dialysis.

SALT EXTRACT (GLOBULIN).

The residue from aqueous extract was extracted by shaking for successive periods of two hours five to six times with 2 per cent salt solution, allowed to settle, and the supernatant liquid was decanted off. The process was repeated twice and the total extract was freed from suspended impurities by centrifuging in a powerful supercentrifuge (36,000 r.p.m.).

The globulin was precipitated from the extract by half-saturation with ammonium sulphate. The globulin was then separated from the clear filtrate by centrifuging, washed with water, dissolved again in salt solution, and then dialysed for four days in a current of tap water and then in a current of distilled water. The precipitated globulin was then successively washed with alcohol and ether, dried, and preserved. The amount of globulin was, however, very small.

It is interesting to note that the aqueous extracts of both hilsa and ruhee contained a high percentage of globulin (salt-soluble protein). This may be

accounted for by the presence of a large amount of salt distributed in the body of the fish. So from the water extract almost all the globulins were obtained and analysed.

CAUSTIC SODA EXTRACT (GLUTELIN).

Since the alcohol extracts contained a very small amount of protein, extraction with this solvent was not carried out.

The residue after the salt extraction was shaken five to six times with 0.2 per cent caustic soda solution for two hours on each occasion. The supernatant liquid was syphoned off and then freed from insoluble residues by centrifuging in a supercentrifuge. The glutelin was precipitated from the clear extract by making the solution slightly acidic with acetic acid. The precipitate was separated, dissolved in 0.2 per cent alkali and again precipitated by acetic acid. The process was repeated several times. A small amount of prolamine coming with the glutelin was removed by shaking the final glutelin with 70 per cent alcohol. The protein thus extracted was washed with absolute alcohol and ether and preserved for analysis.

ANALYSIS OF THE PROTEINS.

The globulin and glutelin from both the varieties of fish were analysed by the van Slyke (1911, 1915) method as modified by Plimmer and Rosedale (1925). The results are indicated in Table II.

Tyrosine and tryptophane were estimated colorimetrically by the method of Folin and Marenzie (1929). Cystine was also directly estimated by the colorimetric method of Sullivan (1926, 1929) as modified by Prunty (1933). The results are indicated in Table III.

TABLE II.

Showing the nitrogen distribution of ruhee and hilsa globulins and glutelins.

Nitrogen.	AS PERCENTAGE OF TOTAL NITROGEN.			
	Ruhee globulin.	Hilsa globulin.	Ruhee glutelin.	Hilsa glutelin.
Acid-insoluble humin	1.08	1.39	1.12	1.28
Acid-soluble humin	1.91	1.98	1.31	2.02
Amide	6.42	6.18	12.48	9.81
<i>Di-amino fraction.</i>				
Arginine	16.42	14.01	20.46	18.29
Histidine	5.22	5.04	3.98	3.81
Cystine (corresponding to total sulphur)	1.92	1.84	2.28	1.01
Lysine	4.28	6.92	5.42	5.48
<i>Mono-amino fraction.</i>				
Amino-N of the filtrate	55.87	54.04	47.42	51.65
Non-amino-N of the filtrate	6.51	7.92	5.11	6.54
TOTAL ..	99.63	99.32	99.58	99.89

TABLE III.

Percentage of total nitrogen in the form of tyrosine, tryptophane, and cystine.

Per cent.	GLOBULIN.		GLUTELIN.	
	Ruhee.	Hilsa.	Ruhee.	Hilsa.
Tyrosine ..	4.12	4.59	4.39	3.62
Tryptophane ..	1.51	1.43	1.72	1.38
Cystine ..	1.02	0.49	1.48	1.25

It is seen from Tables II and III that the cystine content of the proteins as estimated by the colorimetric method is only a fraction of that calculated from the total sulphur content of the proteins. The remaining portion of the sulphur contents of the proteins represent methionine, homocystine, and other sulphur compounds which are not estimated by Sullivan's method.

DISCUSSION.

Analyses of the proteins from ruhee and hilsa show that there is a marked difference between them.

The arginine content of both globulin and glutelin of ruhee (16.42 and 20.46 per cent) is slightly greater than those of hilsa (14.01 and 18.29 per cent).

Histidine contents of both globulin and glutelin of ruhee and hilsa are equal.

Ruhee globulin contains less lysine (4.28 per cent) than hilsa globulin (6.92 per cent). Both globulin and glutelin of ruhee possess a higher percentage of cystine (1.02 and 1.48 per cent) than those of hilsa (0.49 and 1.25 per cent). Ruhee glutelin contains more tyrosine (4.39 per cent) than the hilsa glutelin (3.62 per cent).

In the previous paper (Part I of this series, Basu and De, *loc. cit.*) it has been pointed out by feeding experiments on young rats that the nutritive value of the proteins of the hilsa is lower at all levels of intake. The diet containing 5 per cent hilsa proteins is inadequate for growth promotion, whereas appreciable growth results when the level is raised to 10 per cent hilsa proteins and also with 5 per cent ruhee fish proteins. Since tyrosine is not an indispensable dietary component as reported by Womack and Rose (1934), the discrepancy in growth for the proteins of two varieties of fish is very probably due to the difference in the cystine contents of the proteins of ruhee and hilsa.

SUMMARY.

1. The percentage of total nitrogen extractable by different solvents is 96.4 in the case of ruhee and 93.0 in the case of hilsa.

2. Optimum sodium chloride concentration for extraction is 2 per cent solution.

3. From ruhee, water extracts 32.4 per cent, salt extracts 5.2 per cent, 75 per cent alcohol extracts, 3.2 per cent and 0.2 per cent alkali extracts, 55.6 per cent proteins; the corresponding values for hilsa are 26.8, 9.9, 2.6, and 53.7 per cent, respectively.

4. The globulin and the glutelin constitute the greater proportion of proteins in both the cases. The aqueous extracts contain mostly globulin. On cooling the aqueous extracts in the refrigerator small amounts of albumin are obtained in both the cases, but the amounts are too small for analysis. The aqueous extract of ruhee contains small amounts of an albumin coagulating at 45°C.

5. The nitrogen distribution of ruhee and hilsa fish globulin and glutelin has been determined by the method of van Slyke as modified by Plimmer and Rosedale (*loc. cit.*).

6. Tyrosine and the tryptophane contents of the proteins have been determined by the colorimetric method of Folin *et al.* Tryptophane contents of both hilsa and ruhee proteins are equal. Ruhee glutelin contains more tyrosine than the hilsa glutelin.

7. Ruhee fish proteins contain more sulphur containing amino acids than the hilsa fish proteins.

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NUTRITIONAL INVESTIGATION OF SOME SPECIES OF BENGAL FISH.

Part III.

DETERMINATION OF THE VITAMIN-A CONTENTS OF THE LIVER AND BODY OILS OF RUHEE (*LABEO ROHITA*) AND HILSA (*CLUPEA ILISA*) BY THE BIOLOGICAL METHOD.

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PREVIOUS paper of Basu and De (1938a) has indicated the superiority of *Labeo rohita* over *Clupea ilisa* in maintaining nitrogen equilibrium and in promoting growth. The superiority of the ruhee has been found to be due to a difference in the content of sulphur containing amino acids (Basu and De, 1938b) in the two varieties of fish.

It was felt to be interesting to discover if the body and liver oils of the two fishes reveal any difference in the vitamin-A contents. The determination of the vitamins-A and -D contents of the different varieties of Indian fish is a very important problem of nutrition in India, for some of these might prove as good or even more potent sources of these vitamins than cod-liver oil. Chakravorty *et al.* (1933) and Ghosh *et al.* (1934, 1935) determined the vitamin-A content of the liver oils of some common varieties of Bengal fish, both biologically and tintometrically, and found some but no strict agreement between the biological and the tintometric values. Datta and Banerjee (1934) made a study of the vitamin-A content of the body oils of some species of Indian fish by biological and tintometric examination and found a good agreement between the two results.

PREPARATION OF THE OIL.

The liver of ruhee was collected in the early part of February and that of hilsa in the early part of May. The liver oils were prepared by treating the livers with steam and the oil liberated was filtered through anhydrous sodium sulphate and then extracted with ether.

The body oils were prepared by grinding the meal with anhydrous sodium sulphate and then extracting with ether. After evaporation of the ether, the oils were kept in a coloured stoppered bottle. Precautions were adopted to prevent the oxidation of the oils.

TECHNIQUE.

Young rats, 28 to 29 days old and weighing between 35 and 40 grammes at the beginning of the experiment, were placed on a vitamin-A-free basal diet having the composition :—

				Parts.
Purified casein (B. D. H.)	18
Pure rice starch	66
Salt mixture	4
Purified yeast	10

and this was continued till the body store of vitamin A was depleted. The casein was carefully freed from vitamin A.

Vitamin D was supplied by the incorporation of bi-weekly doses of 4 International Units.

When the weight became steady after depletion of the store of vitamin A, each of the animals was placed in a separate cage with a raised screen to prevent access to the excreta. One rat of each litter was left upon the basal diet only until it died from lack of vitamin A, thus serving as a negative control. Some animals were given 4 International Units of the International standard carotene (supplied by the Coonoor Laboratories) daily and thus served as the positive control. The need for a standard group obtaining the standard dose had been demonstrated by Coward (1933). The best and most accurate results are obtained by a comparison of the response of the group of animals obtaining a given dose of oil with the response of another group obtaining a dose of some standard of reference, two tests being carried out simultaneously. The remaining animals were then daily given graded doses of oil in addition to the basal diet. The diluent used was olive oil and the fish oils were administered orally before the basal diet was given.

The length of the test period necessary has been considered by various workers. Sherman and Munsel (1925), to find the vitamin-A activity of tomato, prolonged the test period for eight weeks. Later, Sherman and Burtius (1928) proposed to confine the test period to four to five weeks and this has been recommended by Coward and others. Recently, Coward *et al.* (1933) have demonstrated that the increase in accuracy obtainable by the prolongation of vitamin-A test beyond a period of three weeks is too slight to justify the expenditure of time and labour

which would be involved. In the present investigation the test period lasted for four to five weeks and it was counted from the first day of dosing.

The growth responses in young rats for different doses of oil (both liver and body) are summarized in Table I:—

TABLE I.

Growth response with various doses of vitamin A containing supplements.

Name of the supplement.	Number of rats.	Dose in mg.	Days under experiment.	Increase in weight per rat in g.	Increase per rat per week in g.	REMARKS.
Carotene ..	7	4 units	28	55.2	13.8	Group I
" ..	6	4 "	28	51.6	12.9	" II
Ruhee-liver oil ..	8	2 mg.	28	12.72	3.18	" I
" " ..	7	5 "	28	29.3	7.3	" "
" " " ..	7	10 "	28	48	12	" "
Hilsa-liver oil ..	6	10 "	28 to 35	17.66	4.16	" "
" ..	7	20 "	28 to 35	30.4	6.28	" "
Ruhee-body oil ..	7	10 "	28 to 35	14.8	3.5	" II
" ..	6	20 "	28 to 35	22.9	5.4	" "
Hilsa-body oil ..	6	100 "	28	Nil	Nil	" "

Table II indicates the growth response with 2 mg. ruhee-liver oil, 10 mg. hilsa-liver oil and 10 mg. ruhee-body oil. The average growths in the different groups are represented in the Graph.

TABLE II.

Growth response of individual rats with different oils.

Rat number.	Experimental period in weeks.	Initial weight in g.	Weight after depletion in g.	Final weight in g.	Average increase in weight in g.	Average increase in weight per week in g.	Mean weekly average gain in g.
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Daily dose—2 mg. ruhee-liver oil. Group I.

411	4	49	98	107	9	2.25	3.18
416	4	45	94	110.5	16.5	4.12	
431	4	46	98	112	14	3.5	
432	4	40	81	98.2	17.2	4.3	
433	4	48	84	92	8	2	
419	4	44	99	118	19	4.75	
420	4	49	80	90.1	10.1	2.52	
421	4	41	85	93	8	2	
AVERAGE		45.7	89.8	101.8	12.8	3.18	..

TABLE II—concl'd.

Rat number.	Experimental period in weeks.	Initial weight in g.	Weight after depletion in g.	Final weight in g.	Average increase in weight in g.	Average increase in weight per week in g.	Mean weekly average gain in g.
<i>Daily dose—10 mg. hilsa-liver oil.</i>							
415	4	40	81	99	18	4.5	4.16
425	5	42	96	106	10	2	
426	4	44	95	119	24	6	
431	4	41	85	105	20	5	
449	4	45	89	103	14	3.5	
450	5	42	84	104	20	4	
AVERAGE		42	88.6	107.5	17.6	4.16	..

Daily dose—10 mg. ruhee-body oil. Group II.

444	4	39	89	109	20	5	3.5
445	5	40	95	105	10	2	
450	5	41	98	103	15	3	
453	4	44	84	99	15	3.75	
458	5	54	94	109	15	3	
461	4	38	88	97	9	2.25	
479	4	39	76	98	22	5.5	
AVERAGE		39.2	89.1	102.9	26.7	3.5	.

GRAPH.

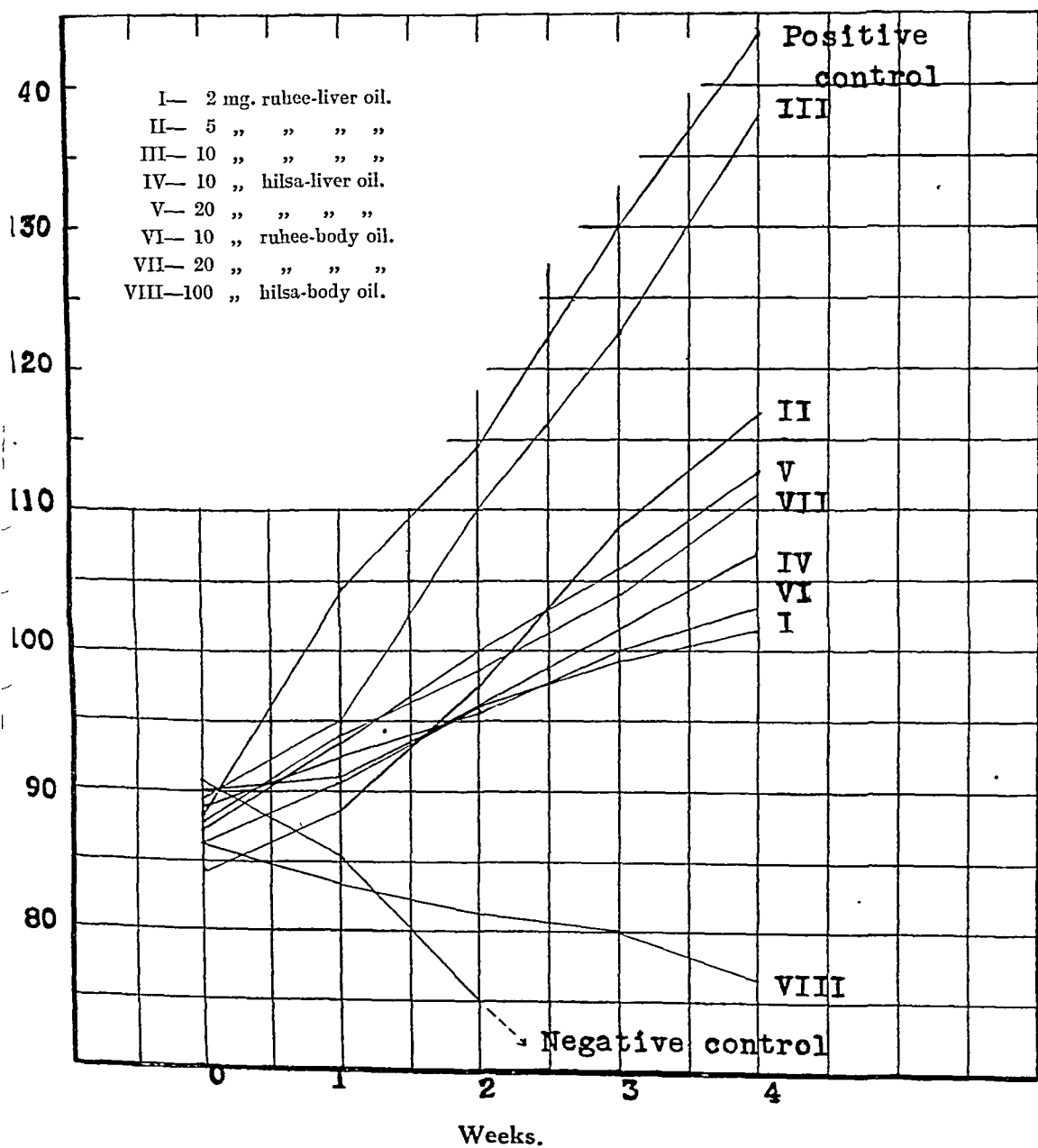


Table III indicates the relative vitamin-A values of the oils under investigation.

TABLE III.

The relative vitamin-A values of different oils.

Name of the oil.	Units of vitamin A per grammc.	Weight of oil (g.) containing one unit.
Ruhee-liver oil ..	461	0.0021
Hilsa-liver oil ..	120	0.0083
Ruhee-body oil ..	109	0.0091
Hilsa-body oil ..	<i>Nil</i>	<i>Nil</i>

DISCUSSION.

Most of the animals showed a steady weight of 80 to 90 grammes after four to five weeks on the basal diet. The majority showed signs of blindness and yellow pigments were observed in the tongues of some.

The negative control of both the groups survived from 13 to 21 days after the period of depletion shown by cessation of growth.

The positive control obtaining 4 units of carotene daily showed a growth response of 13.8 grammes and 12.9 grammes per week in group I and group II, respectively. The relative vitamin-A values for different oils as indicated in Table III are computed by comparison with this sample of carotene and the values are absolute values in terms of International Units.

The liver oils of ruhee and hilsa were found to be potent in doses of 2 mg. and 10 mg., respectively. The body oil of ruhee was found to be potent in the dose of 10 mg., while the body oil of hilsa did not show any growth response even when given in doses of 100 mg. Three out of six rats in hilsa-body oil died after 20 days from the beginning of the test period.

SUMMARY.

1. The vitamin-A potency of the liver oil is higher in the case of ruhee fish than in the case of hilsa fish. The International Units of vitamin A contained in one gramme of the liver oil of ruhee and hilsa are 461 and 120, respectively.

2. The potency of the body oil of ruhee is 109, while that of hilsa is nil.

3. The figures are computed by comparison with a sample of the International standard carotene.

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TABLE II.
Result of the basal metabolism of nine subjects.

		NON-AIR-CONDITIONED ROOMS.							AIR-CONDITIONED ROOMS.						
Number.	Subject.	Age, years.	Bar-pressure, mm.	R. H., per cent.	Temperature, °F.	Respiratory quotient.	Oxygen consumed per minute, c.c.	Heat production per hour, calories.	Heat production per hour per square metre, calories.	R. H., per cent.	Temperature, °F.	Respiratory quotient.	Oxygen consumed per minute, c.c.	Heat production per hour, calories.	Heat production per hour per square metre, calories.
1	C.	19	753	67.0	89.0	0.82	214.9	63.09	39.19	53.0	81.0	0.79	199.2	57.61	35.78
2	H. A.	25	750.5	69.0	87.0	0.81	192.4	56.36	35.67	54.2	79.6	0.75	180.2	52.21	33.04
3	S. M. T.	31	745	76.0	85.5	0.83	230.5	67.8	39.19	67.5	78.5	0.79	209.9	61.28	35.12
4	N. R.	32	746.5	76.0	85.0	0.85	202.2	59.7	32.62	59.0	79.0	0.78	191.1	55.69	30.43
5	A.	22	751.5	74.0	86.0	0.79	217.4	63.47	36.48	69.0	79.0	0.74	213.4	61.71	35.46
6	N.	27	754.5	68.6	86.2	0.79	188.0	54.88	34.3	56.0	79.0	0.72	178.3	51.37	32.1
7	G. R.	27	753	68.5	86.5	0.87	198.7	58.88	36.35	51.5	79.0	0.72	193.3	55.92	34.53
8	M. S.	28	755.6	70.0	85.0	0.83	202.9	59.68	37.3	56.6	77.0	0.81	191.5	56.12	35.07
9	M.	30	753.5	80.0	83.0	0.72	223.7	64.45	37.36	67.0	79.0	0.78	205.1	59.74	31.63

Taking the average experiences the difference in the temperature of the non-air-conditioned and air-conditioned rooms was 6.9°F. and that in the relative humidity was 13.4 per cent. The average lowering of basal metabolism under these circumstances was 6.6 per cent (*vide* Table III). McConnel and Yaglolou (1924) recorded lowest metabolism at the effective temperatures between 75°F. and 83°F. At temperatures lower than 75°F. the basal metabolism slowly rose, while at temperatures above 83°F. the metabolism rose much more sharply.

TABLE III.

Changes observed in the atmospheric conditions and in basal metabolism in the air-conditioned rooms.

Number.	Names.	Temperature, °F.	R. H.	Basal metabolism rate (B. M. R.).	Decrease in B. M. R., per cent.
1	C. ..	-8.0	-14.0	-3.41	8.70
2	H. A. ..	-7.4	-14.8	-2.53	7.10
3	S. M. T. ..	-7.0	-8.5	-3.77	9.61
4	N. R. ..	-6.0	-17.0	-2.19	6.51
5	A. ..	-7.0	-5.0	-1.02	2.79
6	N. ..	-7.2	-12.6	-2.2	6.41
7	G. R. ..	-7.5	-17.0	-1.82	5.00
8	M. S. ..	-8.0	-19.4	-2.23	5.88
9	M. ..	-4.0	-13.0	-2.73	7.30
AVERAGE ..		6.9	13.47	-2.43	6.59

Table IV gives a statement of the results of the basal metabolism of the nine subjects in the present series, as compared with the three American standards. In all cases the basal metabolism of these subjects is lower than that of the American standards. Under the ordinary atmospheric conditions the average metabolism of our subjects is low to the extent of 8.99 per cent, 5.79 per cent, and 6.16 per cent as compared to du Bois, Harris and Benedict, and Dreyer standards, respectively. In the air-conditioned rooms the basal metabolism is very much lower. It is in fact 16.77, 12.07, and 12.44 per cent less respectively as compared to the three standards named above.

TABLE IV.

Deviation in basal metabolism rate in the present series as compared with American standards.

Number.	OPEN ATMOSPHERE.					AIR-CONDITIONED ROOMS.					
	Age in years.	Height, cm.	Weight, kg.	Basal metabolism per hour per square metre.*	Deviation from Aub-du Bois.	Deviation from Harris-Benedict.	Deviation from Dreyer.	Deviation from Aub-du Bois.	Deviation from Harris-Benedict.	Deviation from Dreyer.	Basal metabolism per hour per square metre.
1	19	168.3	54.54	39.19	-4.61	-0.488	-2.18	-14.58	-8.86	-10.68	35.78
2	25	166.7	53.63	35.67	-10.73	-7.81	-8.92	-19.55	-14.68	-15.62	33.04
3	31	163.8	65.9	39.19	-0.80	+2.26	+1.85	-11.52	-7.57	-7.95	35.42
4	32	171.5	70.0	32.62	-21.09	-14.96	-12.82	-29.81	-20.68	-18.67	30.43
5	22	175.9	60.5	36.48	-8.27	-7.31	-5.13	-11.39	-9.88	-7.78	35.46
6	27	166.0	55.45	34.3	-15.16	-11.54	-11.95	-23.05	-17.19	-17.58	32.1
7	27	159.4	60.45	36.35	-8.66	-7.42	-9.52	-14.39	-12.07	-14.07	34.53
8	28	159.4	59.54	37.3	-5.89	-3.12	-7.11	-12.63	-8.89	-12.65	35.07
9	30	169.9	62.3	37.36	-5.73	-1.63	+0.31	-14.06	-8.82	-7.01	34.63
AVERAGE		36.495	-8.99	-5.79	-6.16	-16.77	-12.07	-12.44	34.05

* Air movements were slight and imperceptible in either case.

DISCUSSION.

The ancients ascribe to climate a causative rôle in regard to health and disease. These views prevailed without material alteration till the pre-bacteriological era. With the advent of microbiology and the brilliant successes achieved by bacteriology and allied sciences in solving the mystery of diseases, the views regarding the influence of climate on health swang to the other extreme. However, it is now being realized more and more that the study of climatic conditions should receive greater attention on the part of the health workers than it has done so far. In this country the subject of climate in direct relation to health is particularly important. The health status of an individual must necessarily be considered in terms of the range of adjustability which he possesses in relation to environmental variations. The wider this range is the better are his chances to keep healthy. The present study was originally designed to investigate the variations in the range of adjustability of different individuals in relation to changes in temperature and humidity as measured by an objective test and to determine, if possible, the factors influencing this range. Sufficient progress could not, unfortunately, be made due to lack of facilities. The results presented here will, it is hoped, serve to draw attention to this important problem. While it is not permissible to discuss the significance of the observed changes in basal metabolism under the two sets of atmospheric conditions on the basis of the present data, the observations presented here would appear to suggest that in estimating the efficiency of ventilation it is not enough to consider the rate of loss of heat by the body, but it is necessary to take into account the rate of production of heat as it is definitely influenced by the atmospheric changes in temperature and humidity. It must, however, be clearly understood that these remarks apply only to the circumstances when the subjects are exposed to sudden changes in atmospheric conditions. It is probable that when these changes are slow and take place by degrees, as for instance in seasonal changes, the body adjusts itself to the altered conditions so that no appreciable changes in basal metabolism of individuals occur on that ground.

There is an apparent contradiction in the results obtained in this series of experiments and the recorded higher metabolism in cold countries. We are not in a position to offer any explanation but it is possible that lowering of basal metabolism in the cooler rooms is but a temporary phase and it is followed by a greater reactivity on the part of the body to balance the greater heat loss in temperate climates.

ACKNOWLEDGMENTS.

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INVESTIGATIONS INTO THE EPIDEMIOLOGY OF EPIDEMIC DROPSY.*

Part VI.

ALLYLISOTHIOCYANATE AS AN ÆTIOLOGICAL FACTOR IN EPIDEMIC DROPSY.

BY

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In previous communications (Lal and Roy, 1937a and b) strong evidence was presented to show that certain consignments of mustard oil gave rise to typical symptoms of epidemic dropsy amongst the consumers. In the same reports a suggestion was thrown out that the deleterious substance was not derived from the oils commonly used as adulterants. It was also pointed out that for the solution of the problem of epidemic dropsy from the preventive aspect, it was necessary to develop methods by which batches of oil containing the deleterious substance could be detected and, if possible, the toxic factor could be isolated so that its nature and origin might be investigated.

These problems present many difficulties. The usual practice in Bengal is to purchase only small quantities of oil at a time and, therefore, it is by no means easy to procure oil in sufficient quantities which can be definitely associated with epidemics. Further, as will be seen later, it has not so far been possible to

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reproduce the symptoms of epidemic dropsy in laboratory animals and recourse to human experiments for these investigations has obvious limitations.

The first substance to attract our attention was allylisothiocyanate which is liberated from sinigrin, a glucoside naturally present in the mustard seed, by the action of a ferment known as myrosin, also present in the seed. The allylisothiocyanate content of the oil depends upon the extent of hydrolysis to which the seed has been subjected in the process of expression. In the old *ghani* process hot water is added which favours the liberation of this substance. In the modern types of expellers, the process is dry and little or no break up of the glucoside takes place, hence the addition of synthetic oil of sinapis becomes necessary to increase its pungency and thus its commercial value.

The essential-oil content of the mustard seed is said to vary from 0·8 to 1·23 per cent of which 97 to 98 per cent is allylisothiocyanate. Traces of propylisothiocyanate and allylcyanide have also been detected. So far as we are aware allylisothiocyanate and the allied compounds are the only physiologically active substances naturally present in the oil. Allylisothiocyanate is a powerful rubefacient and may cause blisters on the skin if applied undiluted. The vapour causes profuse watering of the eyes. It has a strong irritant action upon the mucous surfaces. It is not unlikely, therefore, that the oil containing excessive amount of this substance would irritate the mucous membrane of the alimentary tract and cause gastro-intestinal symptoms which are usually the initial manifestations of the epidemic dropsy syndrome. However, the oil cakes which contain the major part of allylisothiocyanate are greedily consumed by the cattle without any ill effects.

In the present investigations we have approached the problem from three aspects, viz.,

(1) The loss of allylisothiocyanate suffered by oil on heating in the process of cooking or otherwise, whether present naturally in the oil or added artificially. This is important because most of the oil is cooked with food before consumption. In the human experiments reported in a previous communication (Lal and Roy, 1937b) the oil was invariably cooked with food.

(2) The effects of large doses of allylisothiocyanate mixed with oil on laboratory animals.

(3) The allylisothiocyanate contents of samples of oil used by a number of families without any ill effects as compared with the concentration of this substance in oil successfully used in human experiments.

THE METHOD OF ESTIMATION OF ALLYLISOTHIOCYANATE.

The methods used for the estimation of allylisothiocyanate in mustard oil was a modification of that adopted by the Association of Official Agricultural Chemists, U. S. A. It may be briefly described as follows:—

Ten grammes of oil were weighed into a flask and 20 c.c. of 95 per cent alcohol were added. The mixture was distilled in steam for half an hour, collecting about 100 c.c. of the distillate into a 250 c.c. volumetric flask containing 10 c.c. of 30 per cent ammonia. Care was taken to see that the end of the condenser dipped below the surface of the solution. Twenty c.c. of N/10 AgNO_3 were added to the

distillate and allowed to stand overnight. The mixture was then heated to boiling over a water-bath for 15 minutes, cooled, made up to 250 c.c., and filtered. One hundred c.c. of the filtrate were acidified with 5 c.c. nitric acid and titrated with 0.01/N ammonium sulphocyanide solution using 3 c.c. of 10 per cent ferric ammonium sulphate as indicator.

One c.c. N/10 AgNO_3 consume = 4.956 mg. allylisothiocyanate.

The results of experiments summarized in Table I serve to show that this method can be relied upon for giving fairly accurate estimation of the amount of allylisothiocyanate present in the oil because known quantities of this substance added to samples of mustard oil freed from the essential oil naturally present (so that no more of it could be recovered) were almost totally recovered in all but one experiment.

TABLE I.

Experiments to test the efficiency of the method for the recovery of allylisothiocyanate.

Serial number.	Nature of sample.	Theoretical quantity of allylisothiocyanate present in mg.	Quantity of allylisothiocyanate recovered in mg.	Percentage of allylisothiocyanate recovered.
1	4 c.c. solution of oil of sinapis ..	38.0	36.67	96.5
2	5 c.c. „ „ „ ..	47.5	46.1	97.1
3*	Mixture of 5 c.c. oil of sinapis and 10 g. mustard oil freed from allylisothiocyanate.	52.5	52.0	99.0
4*	Mixture of 3 c.c. oil of sinapis solution and 10 g. mustard oil freed from allylisothiocyanate.	31.5	30.98	98.3
5*	Mixture of 4 c.c. oil of sinapis solution and 10 g. mustard oil freed from allylisothiocyanate.	47.0	38.7	82.3
6*	Mixture of 5 c.c. oil of sinapis solution with 10 g. mustard oil freed from allylisothiocyanate.	52.5	49.05	93.4
7†	Mixture of 5 c.c. oil of sinapis solution and 10 g. untreated mustard oil.	74.2	72.0	97.0
8†	Mixture of 3 c.c. oil of sinapis solution and 10 g. of untreated mustard oil.	34.64	35.18	100

* The mustard oil used in these experiments was freed from allylisothiocyanate by distilling it in steam for half an hour and heating it to a high temperature under pressure.

† Theoretical value of allylisothiocyanate is the sum of the essential oil added and the amount recovered from the oil by the above process.

I. THE EFFECT OF HEATING THE OIL UPON ITS ALLYLISOTHIOCYANATE CONTENT.

Allylisothiocyanate being a volatile substance one would expect that a major part of it would be lost during the process of cooking. The method of cooking commonly used in the Bengalee homes consists of first heating the oil to a high temperature in a saucepan and then adding the vegetables, curry-powder, etc., and just sufficient amount of water to make the vegetables tender. The time of cooking usually varies from 20 to 40 minutes depending upon the nature of the vegetables.

In Table II is shown the percentage of allylisothiocyanate (naturally present) in mustard oil which is lost when the oil is boiled in water for varying lengths of time. In Table III the results of experiments showing the loss of the essential oil (naturally present) during the actual process of cooking vegetables are summarized. Table IV contains results of a similar experiment conducted with mustard oil to which oil of sinapis had been added. Table V shows the effect of heating the oil, without the addition of water, upon its allylisothiocyanate content.

TABLE II.

Allylisothiocyanate lost when oil is boiled in water.

Sample of oil.	Time of boiling (minutes).	Allylisothiocyanate recovered in mg. from 100 g. of oil.	Percentage of allylisothiocyanate lost.
(1)	0	68.0	0
	5	39.64	41.7
	10	23.3	65.7
	15	5.9	89.0
(2)	0	120.06	0
	5	61.95	48.3
	10	38.00	68.3
	15	15.36	87.5

TABLE III.

Loss of allylisothiocyanate naturally present in oil in the process of cooking vegetables.

Sample of oil.	Time of boiling (minutes).	Allylisothiocyanate recovered in mg. from 100 g. of oil.	Percentage of allylisothiocyanate lost.
I	0	68.0	0
	5	45.84	32.58
	10	24.16	64.47
	15	8.67	87.25

TABLE IV.

Loss of allylisothiocyanate from a mixture of mustard oil and the oil of sinapis in the process of cooking vegetables.

Sample of oil.	Time of cooking (minutes).	Allylisothiocyanate recovered in mg. from 100 g. of oil.	Percentage of allylisothiocyanate lost.
A	0	60.8	0
	5	5.94	88.5
	15	0.989	98.0
B	0	52.65	0
	5	8.67	83.4
	10	5.47	89.5

TABLE V.

Loss of allylisothiocyanate on heating mustard oil and mixture of mustard oil and oil of sinapis in the absence of water.

Nature of sample.	Time of heating (minutes).	Allylisothiocyanate recovered in mg. from 100 g.	Percentage of allylisothiocyanate lost.
A. Jail produced mustard oil ..	0	120.0	0
	5	43.4	64.1
	10	12.4	90.0
B. A mixture of jail produced mustard oil and oil of sinapis.	0	284.2	0
	5	31.0	89.1
	10	6.2	98.6

The results summarized in Tables II, III, IV, and V show that allylisothiocyanate whether present naturally in the mustard oil or added artificially is rapidly lost when the oil is boiled with water or heated as in the process of cooking vegetables. The artificially added oil of sinapis volatilizes much more quickly than the essential oil naturally present. We can, therefore, safely assume that only 10 per cent or less of the total allylisothiocyanate present in the oil is ingested with the food cooked in oil. Is this residual allylisothiocyanate sufficient to cause toxic symptoms? This point is discussed in the next two sections.

II. THE EFFECT OF ALLYLISOTHIOCYANATE ON LABORATORY ANIMALS.

A number of feeding experiments were carried out on three species of animals, namely rats, cats, and monkeys, with oil containing different amounts of allylisothiocyanate. The results of these experiments are given in Tables VI, VII, VIII, and IX.

Experiment 1.

Adult rats were employed. They were divided into five groups of four animals each. The basic diet for all the groups consisted of:—

Atta	69
Casein	10
Salt mixture	4
Yeast	2
Mustard oil	15

Total .. 100

The only difference in the food of the various groups was with regard to the amount of allylisothiocyanate mixed with the oil (*see* Table VI).

GROUP I. Jail produced oil containing 80.8 mg. of allylisothiocyanate per 100 g. of oil, heated without water as in the ordinary process of cooking, before mixing with the basic diet.

GROUP II. * 'S' oil, heated.

GROUP III. 'S' oil, unheated.

GROUP IV. Jail produced oil to which oil of sinapis was added to bring up the allylisothiocyanate content to 300 mg. per 100 g. of oil. The mixture was heated as for group I.

GROUP V. Jail produced oil with oil of sinapis to bring up the allylisothiocyanate content to 700 mg. per 100 g. of oil, heated as in group I.

None of the animals developed any signs of disease suggesting epidemic dropsy. The growth was somewhat better in group I than in group IV. The animals in other groups just maintained their weights. However, these being adult rats much change in weight could not be expected. In order to meet this criticism and to make the experiment more sensitive, it was repeated with young and growing rats.

Experiment 2.

Young rats about a month old weighing 30 g. to 40 g. were used. They were divided into five groups of four animals each. They received the same basic diet as in experiment 1. The description of the oil given is shown against each group.

GROUP I. Jail produced oil, unheated.

GROUP II. 'S' oil, unheated.

GROUP III. † 'K' oil, unheated.

GROUP IV. 'K' oil, heated.

GROUP V. Jail produced oil with oil of sinapis to bring up the allylisothiocyanate content to 700 mg. per 100 g. of oil, unheated.

Details of this experiment are set out in Table VII. It will be noted that judging from the weights there was a slight evidence of toxicity suffered by animals of group V for whom the oil had been highly dosed with allylisothiocyanate, but none of the animals suffered from a condition suggesting epidemic dropsy. Moreover, so far we have not met with a sample of mustard oil which might contain anything like the quantities of allylisothiocyanate artificially added to the oil administered to this group. Half of this quantity is about the maximum limit naturally present in the oil. The loss of hair was common to all the groups and was possibly due to deficiency of flavin. It may also be noted that the animals fed on heated oil consumed a somewhat larger amount of food (as shown under the average weekly consumption of oil) than those fed on unheated oil.

* This oil was recovered from an affected family at Jamshedpur. It was used in human experiments and probably contained the deleterious substance.

† Like 'S' oil this was also obtained from an affected family but the presence of deleterious substance was not tested by human experiments.

TABLE VI.

Experiment I.

Adult rats fed on synthetic diet containing mustard oil to which oil of sinapis was added in varying amounts.

Group.	Rat number.	Sex.	MUSTARD OIL.		Average weekly consumption of oil in g. calculated from food intake.	Total period of feeding in days.	Average weekly change in weight in g.	REMARKS.
			Allylthiocyanate content in mg. per 100 g. of unheated oil.	Heated or unheated.				
I	1	♂	80.8	Heated	7.76	30	+8.5	No symptoms of disease.
	2	♂	80.8	"	7.76	30	+0.25	Do.
	3	♀	80.8	"	6.0	30	+3.5	Do.
	4	♀	80.8	"	6.0	30	+3.25	Do.
II	13	♂	255.2	"	2.55	5	-12.4	Died after five days. Post-mortem not done.
	13 (a)	♂	255.2	"	6.24	26	+1.5	No symptoms of disease.
	14	♂	255.2	"	6.25	30	-2.5	Do.
	15	♀	255.2	"	4.62	30	-1.0	Do.
	16	♀	255.2	"	4.62	30	-3.5	Do.
	17	♂	255.2	Unheated	5.27	26	+0.5	Do.
	18	♂	255.2	"	5.27	26	+1.75	Do.
III	19	♀	255.2	"	4.01	14	-0.75	Looked ill after six days' feeding, killed after one week's illness. Post-mortem: nothing particular.
	20	♀	255.2	"	4.01	26	+1.25	No symptoms of disease.
IV	5	♂	300	Heated	6.63	30	+5.75	Do.
	6	♂	300	"	6.63	30	+3.5	Do.
	7	♀	300	"	5.70	30	+0.75	Do.
	8	♀	300	"	5.70	30	+0.75	Do.
V	9	♂	700	"	7.96	30	+2.5	Do.
	10	♂	700	"	7.96	30	+3.25	Do.
	11	♀	700	"	7.35	30	-2.75	Do.
	12	♀	700	"	7.35	30	+0.75	Do.

TABLE VII.

Experiment II.

Young rats fed on synthetic diet containing mustard oil with varying amounts of oil of sinapis, either naturally present or artificially added.

Group.	Rat number.	Sex.	MUSTARD OIL USED.		Average weekly consumption of oil in g.	Total period of feeding in days.	Average weekly change in weight in g.	REMARKS.
			Allylthiocyanate content in mg. per 100 g. of mustard oil.	Treatment.				
I	21	♂	80.8	Unheated	5.94	47	+3.1	Slight loss of hair first noticed after four weeks' feeding.
	22	♂	80.8	"	5.94	47	+4.8	Do.
	23	♀	80.8	"	5.66	47	+3.6	No symptoms of disease.
	24	♀	80.8	"	5.66	47	+3.0	Slight loss of hair first noticed after four weeks' feeding.
II	29	♂	255.2	"	5.34	47	+4.1	Do.
	30	♂	255.2	"	5.34	47	+0.7	Do.
	31	♀	255.2	"	5.71	47	+3.3	Do.
	32	♀	255.2	"	5.71	47	+4.4	Do.
III	33	♂	308	"	5.77	47	+3.1	Do.
	34	♂	308	"	5.77	47	+4.7	No symptoms.
	35	♀	308	"	5.42	47	+3.4	Slight loss of hair first noticed after four weeks' feeding.
	36	♀	308	"	5.42	47	+2.6	Do.
IV	37	♂	308	Heated	6.5	47	+5.1	Do.
	38	♂	308	"	6.5	47	+6.4	Do.
	39	♀	308	"	6.94	47	+5.3	No symptoms.
	40	♀	308	"	6.94	47	+5.0	Slight loss of hair after five weeks' feeding.

TABLE IX.

*Experiment IV.**Monkeys fed on food containing mustard oil of different types.*

Group number.	Monkey number.	Sex.	Allylisothiocyanate content in mg. per 100 g. of oil.	Heated or unheated.	Average weekly consumption of oil in g.	Period of feeding in days.	Average change in weight in g. per week.	REMARKS.
I	1	♀	80.8	Heated	80.0	30	-56	No sign of disease.
	2	♀	80.8	„	79.1	30	-14	Do.
II	3	♀	300	„	70.77	30	0	Do.
	4	♂	300	„	73.11	30	+70	Died eleven days after stopping the special feeding. Post-mortem tuberculosis of lungs
III	5	♂	700	„	63.13	30	-112	No sign of disease.
	6	♀	700	„	67.51	30	0	Do.

The unsatisfactory progress in the growth of the animals was probably due to the fact that they did not like the food.

III. A COMPARISON OF THE ALLYLISOTHIOCYANATE CONTENT OF SAMPLES OF MUSTARD OIL CONSUMED BY CERTAIN FAMILIES IN CALCUTTA WITH A SAMPLE USED IN FEEDING EXPERIMENTS ON HUMAN SUBJECTS.

As mentioned before, with most people in Bengal it is customary to buy their supplies of mustard oil in small quantities and at short intervals. This circumstance makes it difficult, in a field study, to estimate the effect of mustard oil of a particular description on the health of the consumers. We were, however, able to discover a few families (*vide* Table X) whose usual practice was to purchase their stores for a fortnight or a month at a time. Samples of mustard oil were collected from these families and quantitatively examined for allylisothiocyanate. Information was also obtained regarding the number of persons in the family, their age and sex distribution as well as the amount of oil used and the period in which it was consumed. Inquiries were also made to ascertain if subsequent to the consumption of the oil there had been any sickness in the family which might resemble epidemic dropsy.

As will be seen from Table X, the allylisothiocyanate content of the samples varied from 136 mg. to as high as 272 mg. per 100 g. of oil, the average being

179.5 mg. for the 'Kulu' pressed oil and 222.9 mg. for the mill oil. The period of consumption varied from 12 to 30 days or an average of nearly 20 days. The average amount of oil consumed per individual in terms of per adult per week varied from 130.4 g. to 419.6 g. For this calculation, adjustment of food intake for age and sex was made according to the earlier International scale of family co-efficients (League of Nations' Health Organization, 1932). Taking into consideration the period of consumption of the oil and its allylisothiocyanate content, the total amount of essential oil present in the mustard oil consumed per male adult varied from 672.7 mg. to 2,517.0 mg. None of the members of these families was attacked with epidemic dropsy. Thus an individual may consume mustard oil containing 2,517.0 mg. of allylisothiocyanate within a period of 30 days without showing symptoms of epidemic dropsy. The oil obtained from an affected family at Jamshedpur, in which four out of ten persons were attacked with epidemic dropsy subsequent to the consumption of the oil, had only an allylisothiocyanate content of 136 mg. per 100 g. This oil was used in our human experiment No. 3 (Lal and Roy, 1937b). In this experiment the amount of allylisothiocyanate in the oil consumed by the subjects, all of whom developed symptoms of epidemic dropsy, varied from 50.3 mg. to 672.4 mg. These figures are, however, not of much significance, because the volunteers had previously received oil from a different source for 12 days, the essential oil content of which had not been determined. Most of the subjects had by this time manifested some of the symptoms of the disease. We are, therefore, not in a position to estimate the potency of the experimental oil whose allylisothiocyanate content has been determined. However, there is some evidence to show that this oil had something to do with the causation of the disease, because the three volunteers, who stopped the experimental diet when the supply of the previous oil had been exhausted, developed much milder symptoms than those who continued taking food containing the oil from the new source.

TABLE X.

The allylisothiocyanate content of samples of mustard oil obtained from different families.

Sample number.	Number of persons in the family.	Source of the sample ('Kulu' or mill).	Approximate period for which the sample examined was consumed in days.	A rough estimate of the average quantity of oil consumed in g. per adult male per week.	Quantity of allylisothiocyanate per 100 g. of oil in mg.	Total allylisothiocyanate content of the oil consumed in mg. per adult male.	REMARKS.
1	28	Mill	30	221.1	260	2,464.8	Five members suffered from epidemic dropsy (at Benares) in 1935. All of them partook of the oil examined for four weeks.

TABLE X—*concl.*

Sample number.	Number of persons in the family.	Source of the sample 'Kulu' or mill).	Approximate period for which the sample examined was consumed in days.	A rough estimate of the average quantity of oil consumed in g. per adult male per week.	Quantity of allyliso-thiocyanate per 100 g. of oil in mg.	Total allyliso-thiocyanate content of the oil consumed in mg. per adult male.	REMARKS.
2	7	Mill	21	158.8	210	996.7	..
3	13	'Kulu'	21	294.8	198	1,750.4	..
4	15	Mill	21	130.4	272	1,062.4	Four members gave history of epidemic dropsy in 1935.
5	9	Mill	30	306.2	192	2,517.0	..
6	11	Mill	15	294.8	268	1,692.5	Five members had epidemic dropsy in 1935.
7	13	Mill	30	164.4	241	1,699.2	..
8	4	'Kulu'	15	331.7	136	964.9	..
9	16	Mill	15	348.7	167	1,247.6	History of epidemic dropsy in the family in 1935.
10	2	Mill	21	419.6	173	2,176.2	..
11	5	'Kulu'	15	309.0	185	1,223.7	..
12	3	'Kulu'	21	252.3	231	1,746.4	..
15	19	'Kulu'	12	212.6	185	672.7	..
16	12	'Kulu'	15	223.9	142	679.5	The whole family suffered from epidemic dropsy in 1927.
17	10	Mill	15	155.9	136	454.3	This oil was used for feeding human volunteers.

Three samples of oil obtained from unaffected families of Calcutta (*vide* Table X) were tested for the rate of volatilization of the allylisothiocyanate on boiling it in water with a view to ascertaining whether or not they contained any oil of sinapis added artificially (*vide* Table XI).

TABLE XI.

Rate of volatilization of essential oil contained in certain samples of mustard oil.

Number of sample.	Time of boiling (minutes).	Essential oil recovered from 100 g. of oil in mg.	Percentage of allylisothiocyanate lost.
1 {	0	260.38	0
	5	148.68	42.9
4 {	0	272.56	0
	5	164.04	39.7
	10	121.9	55.8
	15	43.36	84.1
6 {	0	268.77	0
	5	142.48	43.1

These results when compared with those set out in Tables II, III, and IV suggest that the samples did not contain any synthetic allylisothiocyanate.

SUMMARY.

1. Estimations have been made of the loss of allylisothiocyanate suffered by mustard oil when boiled with water, without the addition of water, and when cooked with vegetables.

2. Rates of volatilization of allylisothiocyanate naturally present in the oil and of that artificially added have been estimated.

3. The effects of feeding laboratory animals with food containing mustard oil from different sources with or without the addition of synthetic oil of sinapis have been studied.

4. Allylisothiocyanate content of samples of oil obtained from a number of unaffected families has been estimated and compared with that obtained from an affected family and used in a human experiment.

CONCLUSION.

1. Nearly 90 per cent of allylisothiocyanate content of mustard oil is lost during the process of cooking. The loss is still greater in the case of oil of sinapis added artificially to mustard oil. In the latter case, the rate of volatilization is higher than in the former.

2. Attempts to produce symptoms resembling epidemic dropsy in monkeys, rats, and cats by feeding them with mustard oil from various sources with or without the addition of large doses of oil of sinapis were unsuccessful.

3. Human subjects may take food cooked in oil containing 2,517 mg. of allylisothiocyanate during a period of 30 days without suffering from any ill effects.

4. On the whole, the evidence presented suggests that the deleterious substance in the mustard oil which might produce symptoms of epidemic dropsy is not allylisothiocyanate.

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THE VIRUS OF SANDFLY FEVER IN CULTURE AND CERTAIN OF ITS PROPERTIES.

BY

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In a previous publication Shortt, Rao and Swaminath (1936) gave an account of the cultivation of the virus of sandfly fever on the chorio-allantoic membrane of embryo chicks. The present account may be looked on as a continuation and extension of the work there described.

MATERIALS AND GENERAL TECHNIQUE.

The sandfly-fever sera were sent to us by post from the Military District Laboratory, Peshawar, a train journey of about five days. The sera in any one batch sent us had been collected over a period of about one month and preserved in the interval, in cold storage.

The technique followed for inoculation of the eggs was the standard method used in this laboratory as described by Rao, Pandit and Shortt (1936). A slight modification was, however, introduced in making the actual inoculation, consisting in the addition of a small quantity of normal human serum to the virus suspension. In practice, the lesions were ground in papain-digest broth and to the suspension 10 per cent of normal human serum was added. This mixture was used for inoculations.

This modification appeared to give better lesions than the virus suspension without serum. Besides cultivation on the chorio-allantoic membrane, the virus was grown in tissue culture in flasks. An account of this in more detail, as it has not previously been described by us, is given in subsequent sections. For filtration work Ælford's gradocol membrane technique was employed throughout. Any further details of technique are mentioned in the account which follows.

RESULTS OF CULTURAL INVESTIGATION OF SANDFLY-FEVER SERA.

(a) *On the chorio-allantoic membrane.*—In all, the sera of twenty-seven individuals were available, sent in two batches of seventeen and ten sera respectively.

As our supply of suitable eggs during the hottest months of the year in Madras, which correspond with the sandfly-fever season on the Indian Frontier, is limited we were unable to cultivate the virus in each serum individually. Our usual practice was to pool two sera, the lesion resulting from each pool being given a serial number for future reference in subsequent passages. Sub-passages were made at intervals of four days. The results of the cultivation experiments on the chorio-allantoic membrane are given in Table I :—

TABLE I.

Showing the results of cultivation of the virus of sandfly fever on the chorio-allantoic membrane of embryo chicks.

Serum series.	*Age of serum in days.	Number of eggs used.	†Number of eggs showing lesions and degree of lesion.	Number of passages to date in series where passages were continued.	REMARKS.
1	11	5	3 (+, +, +, focal lesions)	3	Passages stopped on account of negative result.
2	11	6	0
3	13	7	1	5	Passages stopped on account of negative result.
	16	..	(+, focal lesions)	..	
4	20
	20	4	0
5	18
	23	4	1	2	Mixed with series 7.
	26	..	(+ +)
6	22
	29	6	0
7	20
	29	4	3 (++, ++, ++, focal lesions also.)	30	Passages are being continued.

* Time elapsing between drawing of blood and date of culture.

† +, ++, +++ indicate degree of lesion in positive cultures.

TABLE I—concl'd.

Serum series.	*Age of serum in days.	Number of eggs used.	†Number of eggs showing lesions and degree of lesion.	Number of passages to date in series where passages were continued.	REMARKS.
8	21
	29	6	0
9	23
	27	5	0
10	8	7	4 (+++ , ++ , ++ , +)	7	Passages stopped on account of lack of eggs.
11	12
	21	5	3 (+ , ++ , +++)	25	Passages are being continued.
12	33
	33
	34	6	0
13	34
	36
	36	2	2 (++ , +)	11	Passages are being continued.

* Time elapsing between drawing of blood and date of culture.

† + , ++ , +++ indicate degree of lesion in positive cultures.

From the above table it will be seen that out of thirteen pooled batches of sera (generally two in each pool) seven gave a growth of virus and six were negative. The average age of the sera in the negative series was about four days older than in the positive series. In two series, the strains died out after three and five passages respectively. The fact emerges that the virus, as contained in blood serum, is capable of conservation for a month in cold storage, even when there is an intervening railway journey of five days at a high temperature. It is probable that the virus could be conserved for a much longer period if kept at a low temperature continuously.

(b) *In tissue culture.*—As regards cultivation of the virus in tissue culture in flasks, the details of the method used by us, although they were on conventional lines, are given below for the sake of completeness. A ten-day old chick embryo,

after removing its eyes, was finely minced, 0.25 c.c. of Tyrode solution and 0.5 c.c. of virus suspension were added and the whole well mixed and allowed to stand at room temperature for 15 minutes. The mixture was distributed evenly into eight Erhlenmeyer flasks of 50-c.c. capacity, fitted with muslin plugs, and to each was added 0.5 c.c. human serum and 4.0 c.c. Tyrode solution.

As a variation to this technique, we subsequently adopted the practice of adding chick plasma to the cultures. Plotz (1937) observed that virus growth is enhanced if the minced tissue is helped in its proliferation by the presence of plasma clot as a stabilizer and we found this method most satisfactory. To each flask was first added 0.5 c.c. of chick plasma which was spread over the bottom of the flask. To this there were added, in succession, 0.5 c.c. of human serum and the minced chick tissue mixed with virus suspension, while the flasks were agitated gently to ensure even distribution of the fragments of tissue. Clotting quickly took place and then 4.0 c.c. of Tyrode solution were added to each flask.

Whichever of the two techniques described was used, the flasks were left undisturbed at 37°C. for five days. Sterility was then tested for, the contents finely ground, after rejecting about half the fluid contents, and the pooled material from all the flasks used for sub-passages.

By the use of this technique it has proved possible to cultivate the virus direct from sandfly-fever serum and from cultures of the virus on the chorio-allantoic membrane. The virus from flask cultures can also be transferred successfully to the chorio-allantoic membrane. This transfer, in fact, is one means of proving the actual presence of virus in the flask cultures. By direct transfer from flask to flask the virus has been carried, up to date, through eighteen passages.

NEUTRALIZING POWER OF SANDFLY-FEVER ANTISERA ON THE CULTURED VIRUS.

The mere production of lesions on the chorio-allantoic membrane by sandfly-fever sera was not necessarily a proof that the sandfly-fever virus was the lesion-producing agent. It was considered that the presumption in this respect would be increased if it could be shown that the serum of patients, convalescent from sandfly fever, was able to neutralize the lesion-producing agent in the lesion material. To investigate this question the sera of the cases from which the original material for culture was obtained were collected, added to the presumably virus-containing suspensions of the lesions on chorio-allantoic membrane and the mixture inoculated into fresh eggs. The details of the procedure followed are here given.

The pooled membrane lesions of one strain of virus were ground in Tyrode solution in the proportion of 2.0 c.c. of Tyrode solution to each membrane. To 0.5 c.c. of the suspension so obtained, an equal amount of undiluted convalescent serum was added. The mixture was kept at room temperature (85°F.) for 30 minutes and then used in 0.15 c.c. amounts to inoculate eggs. As controls similar mixtures using normal human serum were put up. In addition, to prove the absence of virus in the convalescent sera themselves, these were inoculated into eggs by the usual technique and proved negative.

The details of this experiment are given in Table II:—

TABLE II.
Showing the neutralizing effect of sandfly-fever convalescent serum on the cultured virus of sandfly fever.

SOURCE OF ORIGINAL VIRUS.		Approximate number of days since recovery of cases.	RESULT OF CULTURE ON THE CHORIO-ALLANTOIC MEMBRANE.		CONTROLS WITH NORMAL SERUM PLUS VIRUS.		Convalescent serum control for presence of virus.
*Series.	Passage.		Number of eggs inoculated.	Number of eggs positive.	Number of eggs inoculated.	Number of eggs positive.	
VII H + S	17	85	3	0	4	4	Negative.
XI E + H	12	76	3	0	3	3	Do.
XIII A + M + P	11	96	4	0	5	4	Do.
VII H + S	18	102	5	3	4	3	Do.
XI E + H	13	94	3	0	3	3	Do.
VII H + S	18	106	3	0	5	3	Do.

* The letters indicate the initials of the patients from whom the blood was obtained.

It will be seen from Table II that out of six convalescent sera tested, all but one completely inhibited growth of the virus on the chorio-allantoic membrane. The controls with normal human serum all showed growth of the virus, while the convalescent sera by themselves gave no growth. In series VII H + S the convalescent serum (I) failed to prevent growth of the virus. This serum when originally cultured for virus gave only a poor lesion, graded one +, and died out after one passage. On calling for the clinical history of this case, however, it was not found to differ in any way from the usual course of cases of sandfly fever and the poor immunity response is, therefore, not explained.

Another point brought out by the table is that the virus-neutralizing power of the convalescent sera is not limited to action against the homologous strains of virus since the convalescent sera are in no series homologous to more than one strain in any of the groups of pooled viruses comprising the series in the table.

MORPHOLOGY OF THE VIRUS LESIONS ON THE CHORIO-ALLANTOIC MEMBRANE.

The morphology of the lesions on the chorio-allantoic membrane has been described by Shortt, Rao and Swaminath (*loc. cit.*). A further study of lesions produced on this membrane by a series of the most varied agents (Pandit, Rao and Shortt, 1938) has shown that the reaction of the membrane does not vary greatly, whatever the exciting cause, and that the lesions produced by the same agent may, while maintaining the general form, vary in one particular or another on different occasions. We were not surprised, therefore, in the present series of lesions to find, in many cases, typical and very well developed 'cell nests' such as were described by Shortt, Rao and Swaminath (*loc. cit.*), in lesions produced by the dengue virus and which we now know to occur in lesions produced by various other exciting agents, even when non-viral in nature. These 'cell nests' were the results of ingrowth of the proliferated ectodermal layer. A feature seen in sections of some of the lesions was certain sharply-defined spherical or oval inclusions in the cells of the proliferated ectoderm. These were stained reddish purple both with Mann's stain and with Giemsa and are also brought out as dark bodies by iron-haematoxylin stain. They varied in size from a maximum of about 3.6μ down to mere dots. We do not here use the term 'inclusion' in its special sense as applied to inclusion bodies in various virus infections. We have no reason to suppose that these reddish inclusions are visible aggregates of invisible virus elementary bodies. The same opinion applies to many of the so-called inclusion bodies in other virus infections where they have been looked upon as the virus made visible by local aggregation. They may quite as readily be products of the cell itself, degenerative or otherwise, representing its reaction to the virus or its toxic products.

ANIMAL INOCULATIONS.

A few attempts have been made to infect animals with the virus by various routes, intracerebral, intranasal, intraperitoneal, and subcutaneous.

Intracerebral inoculations.—Two mice were inoculated intracerebrally with 0.03 c.c. of virus suspension strain $\frac{S F III}{3}$. One of these showed symptoms on the seventh day in the form of one-sided paralysis causing circular movements with the head thrust to the left side. Cerebral irritation was indicated by restlessness and incessant scratching of the nose and movements of the front legs. The coat was staring. The brain was passaged into four mice. One showed symptoms on the seventh day similar to those shown by the mouse inoculated from the original material. The brain of this was passaged into four mice. One was found dead on the fifth day having shown no signs of illness the night before. The brain, after filtration, was passaged into two mice without result. Four mice were inoculated with each of three other strains of virus. These remained healthy for three weeks in each case. In one series two mice, although showing no symptoms, were sacrificed on the tenth day and the brains passaged but with no result in an observation period of three weeks.

Intranasal inoculations.—Four mice had instilled into the nose virus suspension of strain F C III. One died on the third day and one on the seventh, without sign of illness previous to death. Seven mice were similarly used with virus strain $\frac{S F VII}{17}$. These remained healthy but two mice were killed on the seventh day, their lungs ground and passaged to four mice by the same intranasal route. Three of these died on the third, fifth and tenth days; as the bodies were putrid no further passages could be made.

Intraperitoneal inoculations.—Two mice were inoculated intraperitoneally with virus of strain $\frac{S F VII}{6}$ and three mice with virus of strain $\frac{F C III}{1}$. All remained healthy for three weeks.

Subcutaneous inoculation.—One mouse was inoculated subcutaneously with virus of strain $\frac{F C III}{1}$. It remained healthy for three weeks.

SIZE OF THE VIRUS.

Suspensions in papain broth of the lesions on the chorio-allantoic membrane were filtered through gradocol membranes of varying average pore diameter. The filtrates, after test for bacteriological sterility, were inoculated into fresh eggs with a view to determining the presence or absence of virus. It was found that a membrane of average pore diameter 0.38μ held back the virus completely, while one of 0.48μ just allowed the passage of the virus. If the diameter of the virus particles be taken as one-third of that of the average pore diameter which will just allow their passage the virus would, from these results, appear to be about $160\mu\mu$ in diameter. The details of one characteristic filtration experiment are given, as an example in Table III.

TABLE III.

Showing details of a filtration experiment.

Average pore diameter of the membrane used for filtration.	Number of eggs inoculated.	Number of eggs positive.	Degree of lesions.	REMARKS.
0.38 μ	6	0	—	minute focal lesions.
0.48 μ	4	2	+, +	
0.6 μ	7	3	+, +, ++	
0.9 μ	6	4	+, +, +, ++	
Unfiltered (control)	7	5	+, +, +, ++, ++	

CIRCULATION OF THE VIRUS IN THE PERIPHERAL BLOOD OF INOCULATED MICE.

The inconclusive nature of the animal experiments led us to determine whether inoculation of the animals would lead to circulation of the virus in the blood and consequent opportunities for distant organs or tissues to become the seat of invasion. It was decided to inoculate mice intraperitoneally with the virus in egg membranes and then to make cultures by the egg technique from brain and heart-blood. Organs such as liver and spleen were avoided owing to their possible direct contamination by the inoculated material.

TABLE IV.

Showing the circulation of sandfly-fever virus in the peripheral blood of inoculated mice.

Mouse number.	Inoculum and route of inoculation.	Date of inoculation.	Interval in days between intraperitoneal inoculation and examination of heart-blood and brain for virus by inoculation into eggs.	RESULTS.					
				HEART-BLOOD.			BRAIN.		
				Number of eggs inoculated.	Number of eggs positive.	Degree of lesions.	Number of eggs inoculated.	Number of eggs positive.	Degree of lesions.
54 {	Pooled virus from	22nd October, 1937	1						
	20th passage group VII								
	15th " " XI			5	4	+, +, +, +	4	4	+, +, +, +, +, +, +
	13th " " XIII								
55 {	0.5 c.c. intraperitoneally	" "	2	6	4	+, +, +, +, +	6	2	+, +, +, +
	Do.								
	Do.			4	3	+, +, +, +, +, +	5	2	+, +, +, +, +
	Do.			4	2	+, +, +, +, +	5	0	-
56 {	Do.	" "	3	4	1	+	5	0	-
	Do.								
	Do.								
	Do.								
57 {	Do.	8th November, 1937	8	4	2	+, +	4	3	+, +, +, +, +
	Do.								
	Do.								
	Do.								
58 {	Do.	" "	3	3	2	+, +, +, +, +	4	2	+, +
	Do.								
	Do.			4	2	+, +, +	4	0	-
	Do.			3	0	-	3	0	-
59 {	Do.	" "	7	4	1	+, +	3	0	-
	Do.								
	Do.								
	Do.								
60 {	Do.	" "	9	4	1	+, +	3	0	-
	Do.								
	Do.								
	Do.								
61 {	Do.	" "	9	4	1	+, +	3	0	-
	Do.								
	Do.								
	Do.								
62 {	Do.	" "	9	4	1	+, +	3	0	-
	Do.								
	Do.								
	Do.								
63 {	Do.	" "	9	4	1	+, +	3	0	-
	Do.								
	Do.								
	Do.								

TABLE V.
Showing the probable identity of the virus in the brain and heart-blood with that inoculated intraperitoneally.

Source of virus.	Serum used in serum-virus mixture.	Final dilution of serum.	Time of contact in minutes.	RESULT.		
				Number of eggs inoculated.	Number of eggs positive.	Degree of lesions.
Membrane lesions from brain material of mouse 54 (undiluted).	Convalescent sandfly-fever serum No. 21.	1 in 2	20	3	1	+
Do.	Normal human serum	1 in 2	20	5	4	++++, ++, ++, +
Membrane lesions from heart-blood of mouse 57 (undiluted).	Convalescent sandfly-fever serum No. 21.	1 in 2	30	4	0	Nil.
Do.	Normal human serum	1 in 2	30	4	4	++++, ++, +, +

Table V clearly demonstrates that sandfly-fever immune serum has a strong inhibitory effect on the virus in the brain and heart-blood of the experimental animals and goes far to confirm the identity of the virus in these situations with that inoculated intraperitoneally.

TRANSPORTATION OF THE VIRUS.

Owing to the inconclusive results obtained in attempts to find a susceptible animal, the co-operation of Dr. G. M. Findlay of the Wellcome Bureau of Scientific Research, London, was sought in the hope that possibly ferrets or hedgehogs—unobtainable by us—might be found susceptible. The virus was mixed with gum acacia, frozen, and dried *in vacuo* and despatched to London by air mail. Dr. Findlay reported its safe arrival and successful culture by the chorio-allantoic membrane technique.

SUMMARY.

1. The virus of sandfly fever has been cultivated by the chorio-allantoic membrane technique and in tissue culture and the cultures carried through numerous passages.

2. The sera of patients recovered from sandfly fever have been shown to possess the power of neutralizing the cultured virus.

3. Certain points in the morphology of the virus lesions on the chorio-allantoic membrane are dealt with.

4. Animal inoculation experiments are described. The inoculation of the cultured virus by the intracerebral, intranasal, intraperitoneal, and subcutaneous routes gave inconclusive results.

5. The size of the virus has been determined to be about 160μ in diameter.

6. The circulation of the cultured virus in the peripheral blood has been demonstrated.

7. The cultured virus has been successfully transported to England by air mail.

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SOME BIOCHEMICAL CHARACTERISTICS OF SNAKE VENOM.*

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THE question as to what constituent or constituents of snake venom might be responsible for the various toxic symptoms manifested has engaged the attention of the workers in different parts of the world since a long time and the volume of literature which has accumulated on this subject is by no means inconsiderable.

The results of broad analysis of the venoms show that they consist of:—

- (1) protein matter (albumin, globulin)—the major portion,
- (2) substances of the nature of proteoses and peptones (not coagulable by heat),
- (3) a colouring matter and an undetermined substance (both soluble in alcohol),
- (4) a trace of fatty matter, and
- (5) inorganic salts (chlorides, phosphates, etc.).

The close resemblance between some forms of poisoning by toxalbuminoids and the snake venom poisoning and the preparation of 'antivenin' by Calmette, which confirmed the toxic nature of the albuminoid fraction, led the earlier workers to believe that this protein molecule was the essential poison; but later experiments showed that it was possible to separate toxic elements of non-protein nature

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from the protein molecule with which they are associated. Faust isolated such a substance from cobra venom which he called ophiotoxin ($C_{17}H_{26}O_{10}$) and from rattle-snake venom a substance called crotalotoxin having the composition ($C_{34}O_{54}O_{21}$) which he believed to be identical with hæmorrhagin, cytolsin, cytotoxin, and hæmolysin. According to him these poisons of venoms were not proteins but glucosides free from nitrogen and belonging to the saponin group of hæmolytic agents. Possibly these glucosides are bound to proteins forming compound proteins which act as specific antigens. Faust also believed that the poisonous elements of different sorts of snake venoms and the dermal poisons of toads and frogs were all closely related substances. The fact that antiserum of one venom fails to protect against another is explained on the assumption that the same or similar poisonous element is attached to different and antigenically specific proteins.

The venoms were known to contain some active enzymes since the latter part of the nineteenth century. Lacerda (1884) (quoted by Wehrmann, 1898) in his lectures on the venom of serpents of Brazil described the results of his researches on the digestive property of venoms. His experiments established that the venoms emulsified fats, coagulated milk, dissolved fibrin, and the coagulated white of egg, but it did not break starches into sugar. Wehrmann (*loc. cit.*) confirmed some of these results. But it is only very recently that workers have begun making these responsible for at least some of the toxic symptoms manifested by the venoms. The work of Mathews (1928) which was later confirmed by Billing (1930) has shown the destruction of fibrinogen by the venom protease to be the principal cause of the failure of the blood to clot in the case of *Crotalus adamanteus* venom although this anticoagulant action is ascribed in part to the splitting of the cephaline molecule rendering it incapable of transforming prothrombin into thrombin. Dunn (1934) also prepared from the venom of *Crotalus adamanteus* an albumose fraction which contains the cephalinase. This fraction is free from heat coagulable protein and forms the substance which imparts yellow colour to the venom. A portion of the toxicity of the original venom is retained by the cephalinase fraction but the toxicity is not parallel to the cephalinase content. The fraction containing the cephalinase has hæmolytic powers of the same order as those of the original venom. This hæmolytic activity does not appear to be due to the cephalinase itself although the venom is capable of producing intensely hæmolytic substances by its action on cephaline.

Considerations such as these led us to investigate the nature and concentration of the different enzymes that may be present in the venoms and also to study other biochemical characteristics in the hope that these might give us some clues for the explanation of some of the more important physiological action manifested by them.

For this purpose we, in the first instance, selected for our study cobra and Russell's viper venoms. Cobra and Russell's viper belong to two different families of snakes, viz., *colubridæ* and *viperidæ* respectively, and though the venoms from these have some characteristics in common, yet they differ also strikingly in other respects. For instance, the neurotoxic element is preponderant in the cobra venom—the main action of this venom in lethal and sublethal doses is on the respiratory centre, the effect being one of initial stimulation and final paralysis;

while hæmorrhagic or anticoagulant property is more or less peculiar to the venom of the viper species. That the nervous centres are not much affected is shown by the fact that in decerebrated animals exactly the same results are produced. So that we thought that by comparing the biochemical properties of these two venoms side by side we might be able to spot out the factors responsible for similarity and disparity in their physiological action also.

It was, therefore, proposed to examine if there is any qualitative or quantitative difference with respect to the protein fractions of these venoms. Table I gives the results of such analysis:—

TABLE I.
Composition of the protein fractions.

Protein fractions.	Cobra venom, per cent.	Russell's viper venom, per cent.
Albumin ..	18·00	22·00
Pseudoglobulin ..	49·00	43·20
Euglobulin ..	Nil	3·60

From the above table it would appear that the viper venom contains more of the albumin but less of the pseudoglobulin portion than that of the cobra; whereas in the cobra venom euglobulin is absent the viper venom contains it to the extent of 3·60 per cent.

The action of various chemicals and some special reagents on these two venoms was next studied but we need mention here only one, viz., the results obtained by treatment with the diazo-reagent according to Clifford and Mottram's (1928) method.

A 2 per cent solution of cobra venom gave a faint reddish coloration but with viper venom of the same strength, a characteristic reddish brown coloration was produced. The susceptibility to this test indicates the probable presence of substances such as carnosine, purine compounds, histamine, etc. This test might have been regarded as very significant had the protein-free venom solutions responded to it, because Russell's viper venom has actually been found to have histamine-like action. This test when applied to protein-free cobra venom solution gave a faint yellow colour, whereas the Russell's viper venom gives a much deeper yellow coloration. We allude to this here only because of this characteristic difference.

We next proceeded to study the nature of the enzymes present in these venoms and to compare the activity of those that are present.

DIASTATIC ENZYMES.

In the ordinary course of things snakes are not likely to be called digest starchy materials, but since the venomous glands of ser ^{are t}

and the venom presents a close analogy with the saliva, we thought that some diastatic ferment, such as ptyaline, might be present.

We, therefore, tested the action of 1.0 per cent solution of these venoms in normal saline upon 0.1 per cent starch solution and incubated them for 24 hours at 40°C. Toluol was used as a preservative.

TABLE II.
Action on starch.

Serial number.	Starch solution in c.c.	Venom solution in c.c.	Time and temperature of incubation.	Cobra readings.	Viper readings.
1	2.0	1.00	24 hours at 40°C.	—	—
2	2.0	0.50	„	—	—
3	2.0	0.25	„	—	—
4	2.0	0.10	„	—	—
5	2.0	0.05	„	—	—

None of these venoms showed the presence of any diastatic ferment. All the tubes of both sets turned deep blue when shaken with one drop of N/50 iodine solution. It was, however, observed that within five minutes the blue coloration of No. 1 tube of each set (i.e., those containing the largest amount of venom) was completely decolorized and in others this decolorization was proportional to the concentration of the venom. In No. 5, i.e., those containing the least amounts of venom, the blue coloration persisted all through. It is probable that the venom solutions have the property of absorbing iodine or of decolorizing starch iodide because on the addition of another drop of iodine solution to the already decolorized solutions, the blue coloration reappeared immediately but this again decolorized after some time.

Invertase action.—Wehrmann (*loc. cit.*) found that venoms possess the property of inverting saccharose. We repeated his experiments but were unable to confirm his observations. No inversion was noticed either polarimetrically or by reductions of Benedict's solution with any of the venoms even when incubated for 24 hours.

Proteolytic enzymes.—The proteolytic enzymes were next studied under the following heads:—

(1) *Digestion of fibrin* (Congo red).—A definite amount of fibrin was placed in each tube and different amounts of the venom solutions were added, the results being recorded according to the intensity of coloration produced.

It would appear from the table that both these venoms have the property of digesting fibrin but the viper venom appears to be somewhat more active in this respect than the cobra. In none of the tubes, however, the fibrin was found to have completely dissolved.

TABLE III.

Serial number.	1.0 per cent venom solution in c.c.	Normal saline in c.c.	Time and temperature of incubation.	Cobra.	Viper.
1	1.00	..	24 hours at 40°C.	+++	+++
2	0.50	0.50	"	++	+++
3	0.25	0.75	"	+	+
4	0.20	0.80	"	±	+
5	0.10	0.90	"	—	—
6	0.05	0.95	"	—	—

(2) *Liquefaction of gelatine*.—A 5 per cent gelatine solution containing 0.1 per cent thymol and 1.0 per cent solution of the venoms in N-saline were used and incubated for 21 hours at 39°C. to 40°C.

After incubation the tubes were placed in ice-cold water for about 15 minutes and in those the gelatine still remained fluid and did not solidify, digestion was supposed to have taken place.

TABLE IV.

Serial number.	Gelatine solution in c.c.	Venom solution in c.c.	Distilled water in c.c.	Cobra.	Viper.
1	1.00	1.00	..	Semi-solid	Liquid.
2	1.00	0.50	0.50	Solid	Semi-solid.
3	1.00	0.25	0.75	"	Solid.
4	1.00	0.10	0.90	"	"
5	1.00	0.05	0.95	"	"
6	1.00	..	1.00	"	"

Here again we find that both these venoms have the property of liquefying gelatine but the viper venoms possess it to a greater extent than the other.

(3) *Rennetic activity* (i.e., property of clotting milk).—The question whether the rennetic activity is due to the existence of a distinct enzyme or is merely the first stage of the manifestation of proteolytic action is still not quite settled, but it appears that the venoms possess the property of clotting milk as well.

Fresh unboiled milk was made fat-free by shaking with chloroform and left in the ice-chest for three days. The residual chloroform was drained out and the milk was freed from chloroform by pouring it in a flat dish and, after gently heating the same on a water-bath, blowing air over it. The milk was found to be amphoteric in reaction. A few drops of toluene was added to each tube as a preservative.

TABLE V.

Serial number.	Amount of milk in c.c.	1 per cent venom solution in c.c.	Distilled water in c.c.	COBRA.		VIPER.	
				3 hours.	21 hours.	3 hours.	21 hours.
1	1.00	1.00	..	Partial	+++	-	+++++
2	1.00	0.50	0.50	—	+++	-	++
3	1.00	0.25	0.75	—	+++	-	±
4	1.00	0.20	0.80	—	+++	-	±
5	1.00	0.10	0.90	—	++	-	—
6	1.00	0.05	0.95	—	—	—	—
7	1.00	..	1.00	—	—	—	—

It may be remarked in this connection that all the tubes which gave positive results showed shrinkage of the casein clot, excepting that containing 1 c.c. of the viper venom, in which the sedimentation was complete and the supernatant liquid was quite clear showing that the action is more intensive in character than in the case of the cobra venom.

(4) *Digestion of casein*.—An 0.5 per cent solution of casein (Hammarsten) was prepared by dissolving 1.5 gramme of casein in 10 c.c. of N/10 NaOH with

gentle heating on the water-bath and diluting to 300 c.c. with distilled water and filtering.

Serial number.	Experiment.	Time and temperature of incubation.	BY VAN SLYKE'S METHOD, C.C. OF N-GAS AT N.T.P.	
			Cobra.	Viper.
1	10 c.c. casein solution + 1 c.c. of 1 per cent venom.	Done immediately	0.22	0.20
2	Do.	24 hours at 40°C.	0.35	0.35

The above results show that the venoms do digest casein but the action is not very marked in either of these.

Therefore, generally speaking, the viper venom appears to be somewhat more active than the cobra venom proteolytically.

Lipolytic action.—We also tested for the presence of lipolytic enzymes in these venoms and found that none of the venoms would split up an emulsion of olive oil. The existence of a lecithin-splitting enzyme in the venoms has been reported by some workers and much importance is attached to this property as it appeared to explain their hæmolytic behaviour and the acceleration of venom hæmolysis by lecithin, but due to the adoption of a faulty technique we could not come to a definite conclusion on this point when this paper was read. Subsequent work with an improved technique, however, has revealed the existence of a very active lecithinase in both the venoms. This will form the subject-matter of a separate communication.

Hæmolysis.—The next point for consideration was the hæmolytic action of these venoms on the R. B. C. of different species of animals. Venoms in general possess the power of dissolving the R. B. C. of some species and lack this property for other species and even with respect to those they do hæmolyse different venoms have different limiting concentrations. The R. B. C. of ox, goat, and sheep are not lysed by any of the venoms, while those of guinea-pig, man, rabbit, mouse, etc., are found to be susceptible in varying degrees. We tested the hæmolytic property of cobra and Russell's viper venoms and found that, while cobra venom hæmolysed the susceptible species of R. B. C. in varying degrees, the viper venom was non-hæmolytic in the large majority of cases. This aspect of the question again will be more fully dealt with in a subsequent communication.

SUMMARY AND CONCLUSIONS.

1. Cobra and Russell's viper venoms have been compared with respect to certain biochemical properties.

2. The viper venom contains more of the albumin but less of the pseudo-globulin portion than that of the cobra. Euglobulin is absent in the cobra venom but the viper venom contains it to the extent of 3.60 per cent.

3. None of the venoms contain any invertase or diastatic enzymes but they both possess the property of digesting fibrin, liquefying gelatine, clotting milk, and also of digesting casein. The viper venom appears to be more active than the cobra venom with respect to proteolytic action.

4. None of the venoms were found to have any appreciable action on an emulsion of olive oil and their action on lecithin was not conclusive, due to the adoption of a faulty technique, though subsequent work has revealed the existence of this enzyme in active condition in both these venoms.

5. Cobra venom is hæmolytic in varying degrees for the susceptible varieties of R. B. C., but the viper venom is non-hæmolytic or hæmolytic only to a very limited extent.

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LIPOLYTIC ACTIVITY OF THE VENOMS (COBRA AND RUSSELL'S VIPER).

BY

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In a previous paper (Roy and Chopra, 1938) cobra and Russell's viper venoms were compared with respect to certain biochemical properties.

It was found that the viper venom contained more of the albumin but less of the pseudoglobulin portion than that of the cobra. No euglobulin was found to be present in the cobra venom but the viper venom contained it to the extent of 3.60 per cent. None of the venoms had any diastatic or invertase activity but they were active proteolytically, viz., in digesting fibrin, liquefying gelatine, clotting milk, and also of digesting casein. The viper venom appeared to be more active than the cobra with respect to this property. The lipolytic activity of these venoms forms the subject-matter of the present communication.

True lipases convert neutral fats to glycerin and fatty acids. It has been found, however, that certain enzymes, while having no action or at least a feeble action upon true fats and oils, readily split the esters of lower fatty acids and mono- or polyvalent alcohols. In addition, there is a definite difference between lipases and esterases on the one hand and the enzymes which act upon the esters of higher alcohols (cholesteryl esters, etc.) or phosphatides (lecithin, cephalin, etc.) on the other. Castor-oil lipase digests true fats actively, while liver and pancreas extracts attack lower esters more energetically. The same Ricinus lipase again split up different fats at different rates. While speaking of lipolytic property, it is therefore important to specify the nature of the substrate or substrates upon which a particular enzyme is found to act.

Kyes (1903) made an important discovery that snake venom forms an actively haemolytic compound with pure lecithin. Morgenroth and Carpi (1906) found the same to be true of the poison of bee. The lipolytic property of various venoms and bee poison, however, was first pointed out by Neuberg and Reicher (1907).

Kyes (1910) applied this observation in explaining the hæmolytic behaviour of the red blood corpuscles of different species of animals towards cobra venom. It is well known that the erythrocytes of certain species of animals such as dog, guinea-pig, man, etc., are lysed by cobra venom in varying degrees, whereas those of ox, sheep, and goat are non-susceptible. According to Kyes, the lecithin in the erythrocytes of the susceptible species is present in a form available for combination with venom, whereas in the non-susceptible varieties the lecithin is supposed to be present in a non-available form. He further believed that the cobra venom formed a true chemical compound with lecithin which he named 'cobra lecithid'.

Noguchi (1907), on the other hand, holds that, although lecithin exists in the stroma of all kinds of corpuscles, it is not present in a form available for venom activation in any of them and the degree of susceptibility to hæmolysis depends chiefly upon the amount of ether-soluble activators present in the cells, as for example, fatty acids, neutral fats, and soluble soaps.

Coca (1912), von Dungern and Coca (1912), and Manwaring (1910) have shown that the venom-lecithin hæmolysis is brought about by the fermentative action of the venom on the lecithin whereby the latter, a non-hæmolytic substance, is split into two parts, oleic acid and the lecithin rest, both of which are strongly hæmolytic. They prefer to call the active principle in cobra venom 'cobra lecithinase' and the complete hæmolysin 'mono-fatty acid lecithin' or 'desoleolecithin'.

According to these workers, therefore, cobra venom does not enter into combination with lecithin in the formation of hæmolysin, but the hæmolysin represents simply one of the split products of lecithin.

It is evident from the above that by 'lipolytic activity' the capacity of a venom in splitting up lecithin alone is implied and we could find no mention in the literature about its property of splitting up other fats and esters and even with respect to the lecithinase activity, as far as we are aware, no attempt for its quantitative estimation has been made.

It was, therefore, proposed to make an investigation with respect to the capacity of cobra and Russell's viper venoms in splitting up (1) true fats such as olive oil, (2) esters formed from lower fatty acids such as ethyl butyrate, (3) lecithin, and (4) cholesterol esters.

(1) *Action on olive oil.*—Kyes and Sachs (1903) pointed out that neutral fats possess the power of favourably influencing venom hæmolysis. He observed that an amount of olive oil (saturated solution of olive oil in methyl alcohol diluted ten times with normal saline), which by itself did not produce any hæmolysis of a suspension of washed ox erythrocytes, will produce complete hæmolysis in the presence of cobra venom. He explained this by assuming that the oil does not act as a direct activator but that it modifies in some way the availability of intracellular lecithin, which is the true activator concerned. As fats are seldom found neutral and as certain amount of free fatty acid is always present, and since higher doses of olive oil itself produce hæmolysis without the simultaneous presence of venom, it was thought that this activation might be due to the free fatty acids liberated by the action of cobra venom on the oil.

The results given in Table I show that none of the venoms has any appreciable action upon neutral fats :—

TABLE I.

	N/50 NaOH required, c.c.
1. 10 c.c. olive-oil emulsion + 5 c.c. N-saline	1·75
2. 10 c.c. ,, ,, + 5 c.c. 1 per cent cobra-venom solution.	3·50
3. 10 c.c. normal saline + 5 c.c. 1 per cent cobra-venom solution.	1·75
4. 5 c.c. olive-oil emulsion + 5 c.c. N-saline	0·50
5. 5 c.c. ,, ,, + 5 c.c. 1 per cent viper venom solution.	2·50
6. 5 c.c. N-saline + 5 c.c. 1 per cent viper venom solution ..	2·20

In the following experiments a saturated solution of olive oil in methyl alcohol was diluted ten times with normal saline and the resulting emulsion was used :—

TABLE II.

				N/50 NaOH required, c.c.
1.	5 c.c. emulsion + 5 c.c. N-saline	0.25
2.	5 c.c. „ + 5 c.c. 1 per cent cobra venom		..	2.25
3.	5 c.c. N-saline + 5 c.c. 1 per cent „ „		..	2.00
4.	5 c.c. emulsion + 5 c.c. 1 per cent viper „		..	2.75
5.	5 c.c. N-saline + 5 c.c. 1 per cent „ „		..	2.50

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It would appear that lecithin does not influence the esterase activity in any manner.

(3) *Lecithinase activity.*—As has already been pointed out, much importance is attached to this property as it is supposed to have an important bearing on the hæmolytic action of cobra venom on susceptible corpuscles and the acceleration of venom hæmolysis in general by means of lecithin.

The procedure previously adopted was to incubate for 24 hours a mixture of lecithin emulsion and venom solution and titrate any fatty acid liberated after the addition of alcohol with phenolphthalein as indicator. The difference in titre between the control (consisting of the same amount of boiled venom solution and lecithin) and the test solution would indicate the activity of the enzyme. Experiments made in this way, however, revealed no appreciable increase of fatty acids in the test solutions. We finally adopted the procedure employed by Dunn (1934) in estimating the cephalinase activity of *Crotalus adamanteus* venom. A measured amount of 1 per cent lecithin emulsion was incubated for 3 hours at 37°C. with the venom solution to be tested. After evaporation to dryness on the steam-bath, the residue was treated with three successive portions of boiling acetone to extract the fatty acids from the unchanged lecithin and lyso-lecithin. The filtered extract was evaporated, dissolved in 95 per cent alcohol, and titrated with N/50 NaOH. The amount of NaOH required for the control was subtracted.

Experiments conducted on this line showed that both cobra and Russell's viper venoms had appreciable lecithinase activity and that the latter had somewhat stronger action.

One per cent emulsion of lecithin was prepared and both cobra and viper venoms in concentrations of 0.5 and 0.05 per cent were allowed to act upon it, one

TABLE VI.

		Lecithin control.	Lecithin control.	Venom 0.05 per cent.	Venom 0.05 per cent.	Venom 0.5 per cent.	Venom 0.5 per cent.
		1	2	3	4	5	6
Incubation period	..	3 hours	20 hours	3 hours	20 hours	3 hours	20 hours
Lecithin emulsion, c.c.	..	15.0	15.0	15.0	15.0	15.0	15.0
Venom solution, c.c.	5.0	5.0	5.0	5.0
N-saline, c.c.	5.0	5.0
Cobra	..	5.0	4.75	5.9	6.25	6.9	6.9
Viper	..	5.0	4.75	6.6	6.75	7.2	7.25

set being incubated for 3 hours and another 20 hours in the incubator at 37°C., suitable controls were run at the same time.

These results show (1) that Merck's lecithin (*ex ovo*) contains appreciable amounts of free fatty acids extractable with acetone.

(2) That both cobra and viper venoms have marked lecithinase activity.

(3) That the viper venom is somewhat more active in this respect than the cobra venom.

(4) That though the lecithinase activity increases with the strength of the venom solution, there is no proportionality between the two.

(5) Period of incubation does not make any marked difference in the titre values.

As venom solutions show appreciable acidity when titrated in the presence of alcohol, we thought of doing some experiments in which boiled venom solutions were used along with lecithin as controls.

The venom solutions in the control flasks were gently boiled over a sand-bath for 15 minutes, and all the flasks after the addition of lecithin were incubated at 37°C. for 21 hours, a little toluene being used as a preservative.

TABLE VII.

		Cobra control.	Viper control.	Cobra.	Viper.	Lecithin control.
		1	2	3	4	5
1 per cent lecithin emulsion, c.c.	..	15.0	15.0	15.0	15.0	15.0
0.5 per cent venom solution, c.c.	..	5.0 (boiled).	5.0 (boiled).	5.0	5.0	..
N-saline, c.c.	5.0
N/50 NaOH required, c.c.	..	6.6	7.5	7.1	7.6	1.5

It is evident, therefore, that there is no marked inactivation of the lecithinase in any case even when the venom solutions are boiled for 15 minutes on a sand-bath. These findings are corroborated by hæmolytic tests. The residues after acetone extraction were tested for solubility. The residues in 1, 2, 3, and 4 were found to be insoluble in ether but soluble in normal saline, whereas that in 5 was soluble in ether but insoluble in normal saline. These were then tested for hæmolytic properties. It was found that even two drops of very dilute solutions of the residues left in the first four tubes hæmolyse sheep's red blood corpuscles almost immediately, without the addition of venom, whereas that containing lecithin alone was hæmolytic only in the presence of venom. It is, therefore, to be inferred that hæmotoxin formation has taken place in all those tubes containing venom solutions boiled or unboiled and that the lecithinase activity is not destroyed by being subjected to a boiling temperature for 15 minutes.

The lecithinase activity was also found to remain almost unimpaired when the tubes containing the venom solutions were kept immersed in a boiling water-bath for 20 minutes.

In another series of experiments, therefore, the control flasks containing the venom solutions were autoclaved for 30 minutes under 15-lb. pressure before addition of lecithin. After the addition of lecithin, the tubes were incubated for 21 hours at 37°C., the acetone extraction and fatty acid determination being carried out as already described. The results are given in Table VIII:—

TABLE VIII.

	COBRA.		VIPER.	
	Autoclaved control.	Unheated.	Autoclaved control.	Unheated.
1 per cent lecithin emulsion, c.c. ..	15	15	15	15
0·5 per cent venom solution, c.c. ..	5	5	5	5
N/50 NaOH required, c.c. ..	2·2	8·95	2·2	9·4

It is evident, therefore, that the lecithinase activity is completely destroyed by this treatment. This is further corroborated by hæmolytic tests carried on with normal saline solution or emulsion of the respective residues left after extraction with acetone. The residues from those containing the autoclaved venom solutions were non-hæmolytic and the other two strongly hæmolytic in the absence of any venom.

Action on cholesterol esters.—To see if any of the venoms has any action on cholesterol esters a 0·5 per cent emulsion of cholesterol oleate was prepared and 0·5 per cent solutions of both cobra and viper venoms were allowed to act upon it at 37°C., for 21 hours, autoclaved venom solutions with the usual amount of esters being used as controls.

TABLE IX.

	COBRA.		VIPER.	
	Autoclaved control.	Unheated.	Autoclaved control.	Unheated.
0·5 per cent cholesterol oleate emulsion, c.c.	5·0	5·0	5·0	5·0
Venom solution, c.c. ..	5·0	5·0	5·0	5·0
N/50 NaOH required, c.c. ..	1·75	2·0	2·50	2·25

It appears that none of the venoms has any appreciable action in splitting up cholesteryl oleate. It was, however, observed that the unheated cobra-venom solution caused appreciable lysis of the cholesteryl ester emulsion so that the emulsion appeared much clarified in comparison with the control in which the ester and normal saline were used. The viper venom, on the other hand, curdles the emulsion, and a flocculent precipitate is formed. The significance of this observation in relation to the hæmolytic action of the venoms will be discussed in a subsequent communication.

SUMMARY AND CONCLUSIONS.

1. The lipolytic properties of cobra and Russell's viper venoms have been compared.

2. None of the venoms has any action on an emulsion of olive oil.

3. Cobra venom acts energetically on ethyl butyrate, but viper venom has no appreciable action upon it.

4. The esterase activity and the hæmolytic activity do not run parallel. The esterase activity is altogether destroyed by heating the venom solution to 56°C. for half an hour, but the hæmolytic property is not appreciably affected by this treatment and it persists though much impaired when heated to 100°C. for 15 minutes.

5. Both the venoms act upon lecithin by splitting up fatty acids, but the viper venom appears to have a somewhat stronger action.

6. The venom lecithinase is very stable and its activity is not appreciably diminished even by subjecting it to a boiling temperature for 15 to 20 minutes. On autoclaving, however, it is entirely destroyed.

7. There is a close parallelism between the lecithinase activity and hæmolysin formation in the presence of lecithin.

8. None of the venoms could split up an emulsion of cholesteryl oleate, but in the presence of cobra venom there was an appreciable lysis of the emulsion, whereas with the viper venom a flocculation of the emulsion was observed.

ACKNOWLEDGMENT.

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IRON-HÆMATOXYLIN STAINING TECHNIQUE: AN ILLUSION.

BY

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SINCE the Heidenhein (1892) method of staining by aqueous solution of hæmatoxylin preceded by mordanting in an aqueous solution of iron-ammonium-alum was introduced it has remained, and still remains, the standard method for permanent preparations of protozoa, especially where it is desired to bring out nuclear structure.

In all textbooks on staining technique great stress is laid upon the importance of using iron-ammonium-alum which shows no signs of decomposition. This can be sufficiently well judged by the appearance of the crystals which should be of a good violet colour. Decomposition of the alum takes place on storage and results in a brown deposit of an insoluble basic alum on the crystals. Such supplies of iron-ammonium-alum which did not contain perfect violet-coloured crystals were considered unsuited for the preliminary mordanting.

It occurred to us that, since the black lake, which is the essential staining element of this method, can be obtained without the use of pure violet crystals, these might not be necessary and a series of experiments to elucidate this point was undertaken.

A solution of iron-ammonium-alum prepared from pure violet crystals will, if kept, gradually produce an insoluble basic deposit which, on shaking the bottle, causes a marked turbidity. This effect is hastened by keeping the solution in ordinary soda-water glass bottles and, to some extent, retarded by keeping it in

Jena glass bottles. Once the change has occurred, however, the solution is usually considered unfit for use and hence freshly prepared solutions are always recommended.

As the change on keeping the solution probably occurs as the result of hydrolysis, the solution increasing in acidity as the basic deposit separates out, it appeared reasonable to suppose that solutions made up in an acid medium would remain clear for a long period and hence, if equally effective for mordanting purposes, would obviate the necessity for always preparing fresh solutions.

To test this hypothesis solutions of iron-ammonium-alum were prepared in 0.5 per cent sulphuric acid and used by the ordinary Heidenhein technique for staining protozoa. These proved very satisfactory and remained perfectly clear during an observation period of several months at room temperature (averaging 85°F.). While making these observations we carried out certain other tests with solutions of iron-ammonium-alum made under varying conditions.

Thus we made 3.5 per cent solution of iron-ammonium-alum under the conditions given below and used these as mordants in staining protozoa by Heidenhein's process:—

1. With good violet crystals in distilled water.
2. With good violet crystals in 0.5 per cent sulphuric acid.
3. With old brown-coloured deteriorated crystals in distilled water.
4. With old brown-coloured deteriorated crystals in 0.5 per cent sulphuric acid.
5. With good violet crystals in 0.5 per cent sodium-carbonate solution, which gave a muddy precipitate.

The staining results could not be differentiated in the five series.

These five solutions were kept for some months and re-tested in a similar manner. Again the result in each case was a perfectly satisfactory stain.

SUMMARY.

1. In staining protozoa by the Heidenhein iron-hæmatoxylin process it is unnecessary to use only violet crystals of iron-ammonium-alum as a mordant.

2. Solutions of iron-ammonium-alum which have produced a precipitate on keeping are satisfactory in use.

3. To make a permanently clear solution of iron-ammonium-alum it may be dissolved in 0.5 per cent sulphuric acid.

4. The only essential factor in producing a good iron-hæmatoxylin stain is a good solution of hæmatoxylin.

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ON SANDFLIES (*PHLEBOTOMUS*) FROM CEYLON, SIAM, AND MALAY.

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THE present paper deals with collections of sandflies made during 1932 to 1934 by Mr. H. F. Carter, and with material sent for identification by Sir Guy Marshal, Director, Imperial Institute of Entomology, and Dr. F. W. Edwards of the British Museum (Nat. Hist.). A part of the work was done in the Department of Entomology, London School of Hygiene and Tropical Medicine. The author is most grateful to Mr. H. S. Sutton of that Institution who carried out much of the preliminary work.

Phlebotomus zeylanicus Annandale.

The female of this species was described by Annandale in 1910. The male was described by Young and Chalam (1927) as *P. chalami* n. sp. Sinton (1928a) recognized *P. chalami* as the hitherto unknown male of *P. zeylanicus*. He also published drawings of the buccal cavity and pharynx of the female and of some parts of the male genitalia (Sinton, 1932, 1933a).

A collection of *P. zeylanicus* forwarded by Mr. H. F. Carter from Ceylon gave an opportunity of studying this species anew and several interesting facts appeared. Among the material examined there were two clearly defined forms of females differing mainly in the structure of the buccal cavity and in the wing index $\frac{a}{p}$, one of which turned out to be the hitherto unknown female of *P. arboris*. The spermatheca of *P. zeylanicus* can now be described. The male genitalia in our material differ in several important details from the descriptions and drawings by Sinton, and by Young and Chalam.

FEMALE.

Size: 2.7 mm. Wings: length 2.35 mm., width 0.7 mm., wing index $\frac{a}{p} = 2.1$ to 2.85. Palpi: formula: 1, 2, 3, 4, 5. Segment 4 only

very slightly longer than segment 3. Relative length of segments: 1—3·3—4·8—5—8. Antennæ: Segment 3 > 4 + 5. $\frac{A\ 3}{E} = 1\cdot1$.

Buccal cavity (Plate V, fig. a).—A straight row of 11 to 18 long pointed teeth and 2 to 4 rows of short, stumpy teeth anterior to the long teeth. Pigmented area rounded anteriorly in the form of half a circle with a short anterior process.

Pharynx.—Lamp-glass shaped; 2 to 3 times as wide posteriorly as in the anterior region, with some rows of very minute teeth in the posterior narrow portion.

Spermathecae (Plate V, fig. f).—Only the apical part could be made out in one specimen. They are apparently wide thin-walled tubes as in *P. minutus* with faint transverse striations.

MALE.

Size: 2·3 to 2·5 mm. Wings: length 2 mm., width 0·6 mm., wing index $\frac{a}{\beta} = 1\cdot5$ to 2·7. Average: 2. Palpi: formula: 1, 2, 3, 4, 5. Relative length of segments: 1—3—4·4—4·6—8. Antennæ: Segment 3 > 4 + 5. $\frac{A\ 3}{E} = 1\cdot3$ to 1·8.

Buccal cavity.—A single row of 8 to 10 teeth, with one or two rows of short, stumpy teeth anteriorly. No pigmented area.

Pharynx.—Similar to that of female but slenderer, with a few rows of small teeth in the posterior narrow part.

Genitalia (Plate VI).—The distal segment of the superior clasper markedly longer than half the basal segment, which bears on its inner surface a broad brush of long hairs. Four spatulate spines on the distal segment, two terminal and two at the middle of the segment. The small ventral seta situated slightly distal to the two middle setæ. The middle clasper with wide rounded head and prominent beak-like process and 6 to 8 long hairs ventrally. Penis short and stout, slightly tapering towards a short point. Inferior clasper long and pointed, slightly shorter than the basal segment of the superior clasper.

The shape of the middle clasper differs from that given by Sinton and by Young and Chalam who show a narrow hooked head without the ventral hairs. The penis is much narrower than that figured by Sinton. The second segment of the superior clasper is longer than half the length of the first segment, while in Young and Chalam's specimen it is shorter than half the length of the first. The inferior clasper is shorter than the first segment of the superior clasper, while it is longer in Young and Chalam's specimen.

The material examined was collected by Mr. H. F. Carter from the following localities in Ceylon:—

Godigomuwa	30-10-1932 to 13-11-1932	71 ♂♂, 9 ♀♀.
Katuwawala	27-11-1932	27 ♂♂, 4 ♀♀.
Depanama Panni	..	19-2-1933 to 25-3-1933	7 ♂♂, 1 ♀.
Maharagama	21-5-1933	6 ♂♂.
Kalagoda	6-3-1934	5 ♂♂, 2 ♀♀.

The number of females recorded above probably includes some females of *P. arboris*.

P. arboris Sinton.

The male of this species was described in 1931 from Darjeeling in Bengal. The specimens were caught together with *P. zeylanicus*.

Carter's collection from Ceylon contained 18 male specimens of *P. arboris*, 116 males of *P. zeylanicus*, and 20 females at first identified as those of *P. zeylanicus*. A more detailed examination of the female specimens revealed among them the presence of two different forms, which were rather similar superficially. They showed however constant differences in size, wing index $\frac{a}{b}$ and in the buccal cavity. A comparison of the measurements of wings, etc., with those of the males of *P. arboris* established beyond doubt that one of the two forms was the hitherto unknown female of *P. arboris*.

MALE.

Size: 1.9 mm. Wings: length 1.7 mm., width 0.45 mm., wing index $\frac{a}{b} = 0.7$ to 1. Palpi: formula: 1, 2, 3, 4, 5. Relative length of segments: 1 — 3 — 4.2 — 4.8 — 8. Antennæ: Segment $3 > 4 + 5$. $\frac{A}{E} = 1.75$.

Buccal cavity (Plate V, fig. d).—A slightly curved row of 9 to 12 broad teeth each of which bears several small secondary indentations. The lateral teeth point towards the centre. Sometimes the teeth are so turned that only their narrow edge is visible and they appear then as simple pointed teeth. There are two or three rows of small stumpy teeth anterior to the row of big teeth. These small teeth are sometimes arranged in groups of two or three. Pigmented area triangular or heart-shaped, pointed anteriorly, covering half the width of the buccal cavity or less.

Pharynx.—Lamp-glass shaped, slender, with some rows of teeth in the posterior narrow part.

Genitalia.—Second segment of the superior clasper with four spines, of which two are terminal and two arise at the apical third of the segment. The small ventral seta is situated at about the proximal third of the segment. Second segment about half the length of the first or slightly longer. Middle clasper with a narrow rounded head. Penis narrow, tapering to a fine point. Inferior clasper slightly shorter than the basal segment of the superior clasper.

This description agrees in all essential points with that given by Sinton, except that he does not mention the rows of small teeth in front of the broad teeth.

FEMALE.

Size: 2.1 mm. Wings: length 1.7 mm., width 0.5 mm., wing index $\frac{a}{b} = 1$. Palpi: formula: 1, 2, 3, 4, 5. Relative length of segments 1 — 3 — 4.6 — 5 — 8. Antennæ: Segment $3 > 4 + 5$. $\frac{A}{E} = 1.15$.

Buccal cavity (Plate V, fig. b).—A straight row of 15 to 19 pointed teeth placed rather nearer to each other than in *P. zeylanicus*. Seven to eight rows of short, stumpy teeth anterior to the big teeth. Pigmented area triangular, stretching over the whole width of the buccal cavity with a long anterior process. Near the apex of the triangle (not of the process) there is sometimes a heavily pigmented definitely outlined cap.

Pharynx (Plate V, fig. c).—Lamp-glass shaped, 2.0 to 2.5 times as wide posteriorly as anteriorly with some rows of minute teeth in the posterior narrow portion.

Spermathecae (Plate V, fig. c).—Smooth elliptical capsules with thin walls and a collar round the anterior process. The ducts could not be made out.

P. arboris differs from *P. zeylanicus* in its smaller size. The wing index $\frac{a}{b}$ is always greater than 2 in the female *P. zeylanicus*, while it is about 1 in the female *P. arboris*. In the male the wing index $\frac{a}{b}$ is always greater than 1.5 in *P. zeylanicus* and 0.7 to 1 in *P. arboris*. The male genitalia of the two species are easily distinguished from each other by the position of the basal pair of spines on the second segment of the superior clasper and by the forms of the middle clasper and of the penis.

In the buccal cavity of the female the long pointed teeth are more widely spaced in *P. zeylanicus* and there are only 2 to 3 rows of small teeth as against 7 to 8 in *P. arboris*. The pigmented area is rounded anteriorly in *P. zeylanicus* and triangular in *P. arboris*. The spermathecae in both species differ as indicated in the drawings. There is no striation in the spermathecae of *P. arboris*. It is, however, very difficult to see the spermathecae in both species and fresh material should be examined in order to define the structure of these organs and particularly of their ducts.

A re-examination of collections of *P. zeylanicus* which were caught together with males of *P. arboris* will probably reveal the presence of the female of *P. arboris* described above.

The distribution of the females of Carter's collection from Ceylon cannot be given completely as some of the material was no longer available when the female of *P. arboris* was recognized.

Material examined was collected by Carter from the following localities in Ceylon:—

Godigomuwa	30-10-1932 to 13-11-1932	4 ♀ ♀, 13 ♂ ♂.
Katuwawala	27-11-1932	2 ♀ ♀, 1 ♂.
Depanama Panni	..	25-3-1933	3 ♂ ♂.
Kalagoda	6-3-1934	1 ♀.

P. babu var. *insularis* var. nov.

P. babu was originally described by Annandale in 1910. Later it was considered to be a synonym of *P. minutus*. Sinton re-established the species after examining the buccal armature which he found to differ from that of *P. minutus*.

P. babu has been found to be widely distributed in India and has since been recorded from Mauritius (Theodor, 1931). Specimens resembling *P. babu* very closely were found among the material from Ceylon. There is no full description of *P. babu* in the recent literature and the specimens from Ceylon are, therefore, described in detail.

FEMALE.

Size: 1.7 mm. Wings: length 1.53 mm., width 0.38 mm. ($\frac{\text{length}}{\text{width}} = 4$). Wing index $\frac{\alpha}{\beta} = 0.65$ to 0.8. The wings of a specimen of *P. babu* from the Punjab are much narrower ($\frac{\text{length}}{\text{width}} = 5$). Palpi: formula: 1, 2 (3, 4), 5. Relative length of segments: 1 — 2 — 2.6 — 2.8 — 4.5. This formula differs slightly from that of *P. babu* from India and Mauritius in which palpal segment 4 is either slightly shorter than, or equal in length to, segment 3. Antennæ: Segment 3 < 4 + 5. $\frac{A^3}{E} = 1$ to 1.15.

Buccal cavity (Plate VII, fig. a).—A row of about 45 to 50 equal teeth lying in an arc which is concave posteriorly. This is the main difference from *P. babu* in which species the number of teeth varies from 25 to 33 in specimens from different localities, e.g., 24 in a specimen from the Punjab (Plate VII, figs. c, d); 30 to 34 in specimens from Mauritius and India (exact locality of these specimens unknown). The notch in the buccal cavity is deep and rounded and relatively bigger in the specimens from Ceylon than in *P. babu*. The pigmented area is faint and broadly heart-shaped with a truncated or sometimes bifid anterior process.

Pharynx (Plate VII, fig. b).—Slender, twice as wide anteriorly as posteriorly, with numerous narrow pointed teeth in the posterior part. In specimens of *P. babu* from India and Mauritius the teeth in the pharynx are much broader, scale-like and the armature extends higher up in the pharynx than in the specimens from Ceylon. This was particularly marked in the specimen from the Punjab (Plate VII, fig. d) in which the teeth are still broader than in other specimens from India (Plate VII, fig. f) and in which the armature extends to about one-third of the length of the pharynx.

Spermatheca (Plate VII, fig. c).—Wide elliptical capsules with a short anterior process and a collar around it. The posterior opening of the capsules is wide and continues into a duct which narrows gradually.

MALE.

No difference could be found between the male specimens from Ceylon and those of *P. babu* from other localities. The genitalia have been figured by Sinton (1933b).

The differences described above, i.e., number of teeth in the buccal cavity, number and shape of teeth in the pharynx and the other small differences, are constant in all the material from Ceylon and are sufficient to separate these sandflies from the type species as a new variety for which the name *P. babu* var. *insularis* is proposed.

Material examined was collected by Carter from the following localities in Ceylon :—

Godigomuwa	13-11-1932	1 ♂.
Pannipitiya	5-2-1933	2 ♀ ♀.
Depanama Panni	19-2-1932	3 ♀ ♀, 3 ♂ ♂.
Maharagama	21-5-1933	3 ♂ ♂, 1 ♀.

P. iyengari var. *malayensis* var. nov.

FEMALE.

Size: 2.1 mm. Wings: length 1.8 mm., width 0.43 mm., wing index $\frac{a}{p} = 1.5$. Palpi: formula: 1, 2, 3, 4, 5. Relative length of segments: 1 — 2.5 — 4 — 4.5 — 10. Antennæ: Segment 3 > 4 + 5. $\frac{A3}{E} = 1.3$.

Buccal cavity (Plate VIII, fig. d).—A row of 15 to 17 long pointed teeth arranged in an arc slightly concave posteriorly. The four median teeth are placed more closely together than the lateral ones. There is a second row of much smaller stumpy teeth anterior to the long teeth. The median ones of these small teeth are pointed and larger than the lateral ones. At the sides there is sometimes a double or triple row of small teeth. In some cases a third row of teeth seems to be present in the middle, corresponding in position to the median teeth of the second row. This, however, is only an impression made by the points of the larger teeth of the second row on the pigmented area which apparently rests on these teeth. The pigmented area stretches over the whole width of the buccal cavity and it is rounded anteriorly with a moderately long pointed anterior process.

Pharynx.—Lamp-glass shaped, twice as wide posteriorly as anteriorly with a few transverse ridges in the posterior narrow part.

Spermathecae (Plate VIII, fig. e).—Long cylindrical capsules with slightly crinkled surface, moderately thick walls, and relatively long anterior process. In some specimens there are traces of irregular internal segmentation.

MALE.

Size: 2.1 mm. Wings: length 1.6 mm., width 0.36 mm., wing index $\frac{a}{p} = 0.9$ to 1.1. Antennæ: Segment 3 > 4 + 5. $\frac{A3}{E} = 1.8$ to 2. Palpi as in female.

Buccal cavity.—A row of 8 to 10 short teeth with a rudimentary second row of still smaller stumpy teeth. No pigmented area.

Pharynx.—Lamp-glass shaped, but slenderer than in the female. No armature except some transverse ridges in the posterior part.

Genitalia.—Basal segment of superior clasper slightly more than twice the length of distal segment. Four setæ on the distal segment, of which two are terminal and two markedly sub-terminal. The small ventral seta is situated at the apical third of the segment. The middle clasper has a pointed beak-like

termination. The penis is gradually tapering with a blunt tip. The inferior clasper is slightly shorter than the basal segment of the superior clasper.

Sinton (1933a) described the female of *P. iyengari* from Travancore (2 ♀ ♀). Raynal and Gaschen (1935a) completed this description and described the hitherto unknown male from Indo-China (1 ♂, 1 ♀). Later in the same year Raynal and Gaschen (1935b) described *P. hivernus* from Indo-China.

The material from Selangore described above is very closely related to *P. iyengari*. Measurements and the form of the buccal cavity and pharynx correspond closely with those of the latter species, except that the number of teeth in the buccal cavity is slightly larger (14 in *P. iyengari*; 15 to 17 in the material from Selangore). Sinton does not mention the presence of a second row of small teeth in the buccal cavity but Raynal and Gaschen describe it. The form of the pigmented area is different, the anterior process is narrower and pointed, and the posterior projection of the pigmented area is absent in *P. iyengari*. The spermathecae are intermediate between those of *P. iyengari* and *P. hivernus* in showing a capsule and some traces of internal segmentation, but resemble those of *P. hivernus* in general form. The wing index $\frac{c}{p}$ is slightly larger (in the material from Selangore) than in *P. iyengari*, but this is a rather variable character.

The male genitalia are slightly different in that two of the setae on the apical segment of the superior clasper are markedly sub-terminal, while all 4 are terminal in *P. iyengari*. The middle clasper ends in a beak-like process, while Raynal and Gaschen describe a blunt finger-like termination (of the middle clasper) in *P. iyengari*, though this may probably have been due to distortion in the only male specimen in their collection. (This is supported by the fact that in their illustration of the male genitalia of *P. iyengari* they have shown the bases of the hairs on the ventral surface of the distal part of the middle clasper, while these hairs are usually dorsal in the *minutus* group).

The differences described above do not apparently justify the creation of a new species but they are sufficient to consider the specimens from Selangore as a new variety for which the name *P. iyengari* var. *malayensis* is proposed.

Material examined: 5 ♀ ♀, 17 ♂ ♂, Selangore, Malay Peninsula ('in old packing case with coco-nut husks'). A. G. Pillai. 4-10-1935.

Phlebotomus iyengari var. *hivernus* Raynal and Gaschen.

A critical comparison of the description of *P. iyengari* with that of *P. hivernus* as given by Raynal and Gaschen reveals a very close resemblance in these two species. The characters on which the new species *P. hivernus* was erected (size, number of teeth in the anterior row in the buccal cavity, the form of the spermathecae) do not seem sufficient for that purpose. The form of the spermathecae which is the main distinguishing character between them seems to vary considerably, as indicated by the form of the spermathecae in *P. iyengari* var. *malayensis*, which is intermediate between those of *P. iyengari* and of *P. hivernus*.

It is, therefore, proposed to consider *P. hivernus* as a variety of *P. iyengari*, until the examination of a sufficiently large series of the three forms, discussed above, brings out further characters for distinguishing them.

P. durenii Parrot is also very closely related to the above three forms, from which it differs in minor details only and it is doubtful whether this also should not be considered as a variety of *P. iyengarii*. But as the male of *P. durenii* is unknown it is premature to discuss its relationship to *P. iyengarii* and its varieties.

***P. barraudi* (?) var. nov.**

One female specimen from Bangkok (Siam) was among the material. The measurements of the various parts and the morphology of the pharynx and spermathecae correspond on the whole to the description of *P. barraudi* given by Sinton. There are, however, some differences in the buccal cavity. The specimen from Siam had 54 teeth (Plate VIII, fig. *a*) and the teeth in the pharynx (Plate VIII, fig. *b*) are much shorter than those shown in Sinton's paper. They are certainly very different from those figured by Raynal and Gaschen (1934*a* and *b*) for this species from Indo-China. The palpal formula of the specimen of *P. barraudi* from Indo-China also seems to be slightly different from that in the Indian form of this species, e.g., the third segment is nearly as long as the fourth in the latter, while it is definitely shorter in the former specimens. It is, therefore, possible that the form from Bangkok and that from Indo-China may have to be made separate varieties of *P. barraudi*.

Material examined: 1 ♀, Bangkok (Siam), July 1932 (Causey).

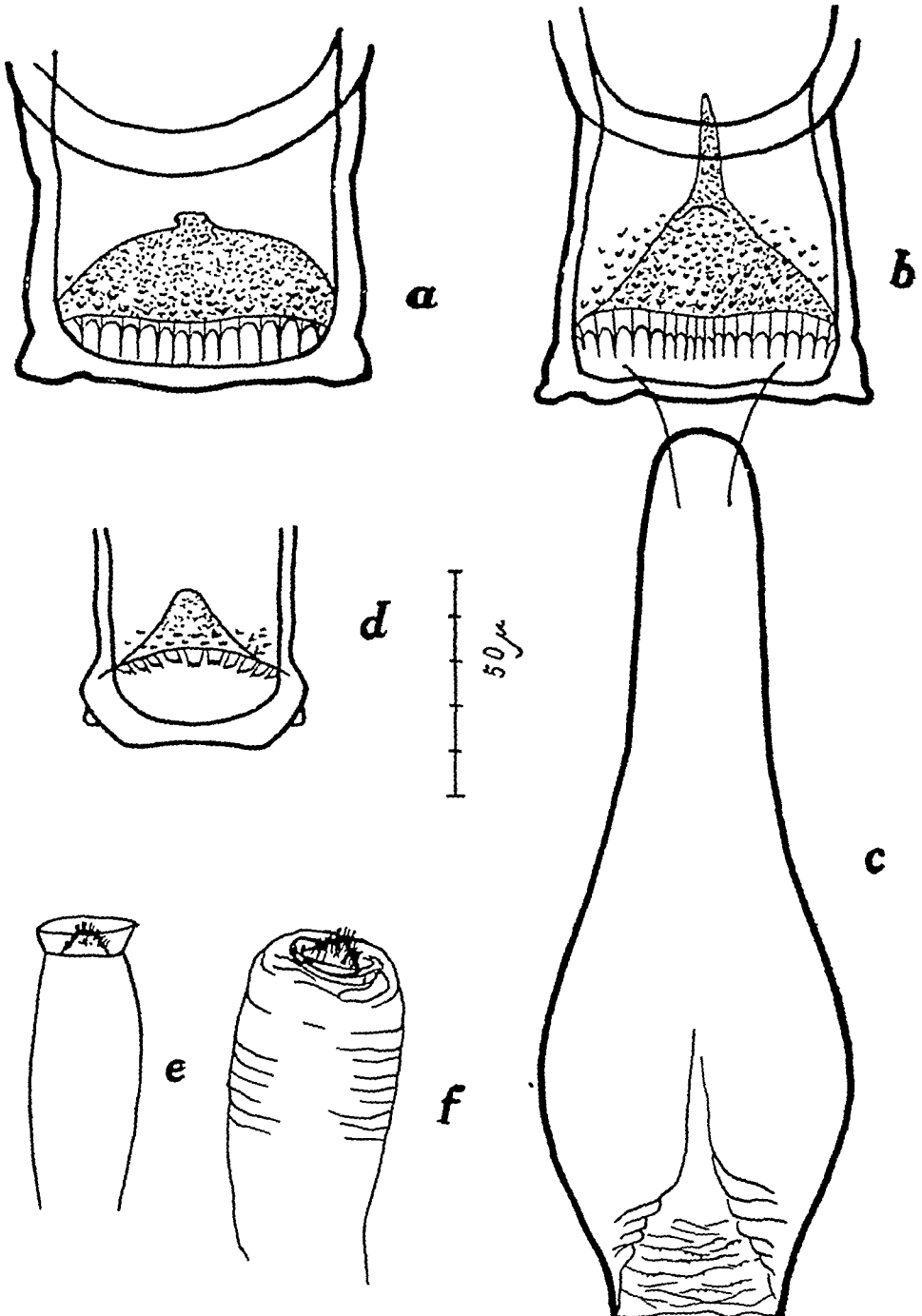
***P. bailyi* var. *campester* Sinton.**

Two female specimens of this variety are in the collection from Bangkok. The measurements of these two females correspond to those given by Sinton, and by Raynal and Gaschen. The arrangement of the teeth in the buccal cavity, however, somewhat differs from that given by Sinton, who shows small teeth of equal size distributed in little groups in two to three irregular lines. In these our specimens from Bangkok the median teeth are markedly bigger than the lateral ones and they are arranged, in one specimen, in a nearly continuous row, with a broken second row at the sides. In the second specimen there is a short row of 5 longer teeth in the middle and some isolated groups in two rows laterally (Plate VIII, fig. *c*). In this specimen the arrangement rather resembles that figured by Raynal and Gaschen.

P. bailyi var. *campester* seems very closely related to *P. nicnic* Banks as pointed out by Sinton (1931*a*). The characters on which Sinton bases the distinction of the female *P. bailyi* var. *campester* from *P. nicnic* are either very variable (expansion of posterior part of buccal cavity, length of third antennal segment) or they are minor characters. On the basis of this information *P. bailyi* var. *campester* might easily be considered a variety of *P. nicnic*, but as there is no material for comparison and the description of the male *P. nicnic* is not available this question is left open.

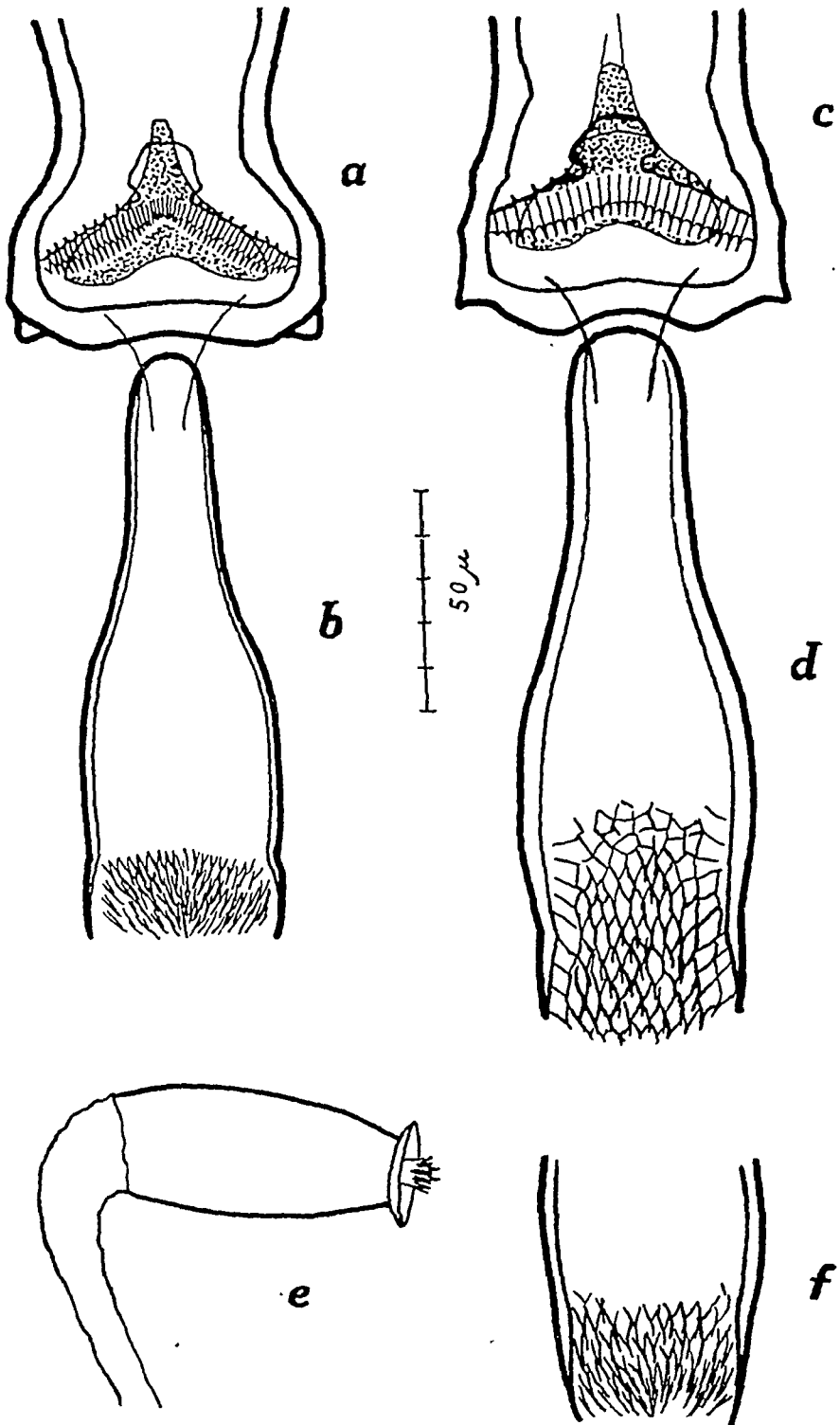
Sinton also records this species from Bangkok.

Material examined: 2 ♀♀, Bangkok (Siam), July 1932 (Causey).



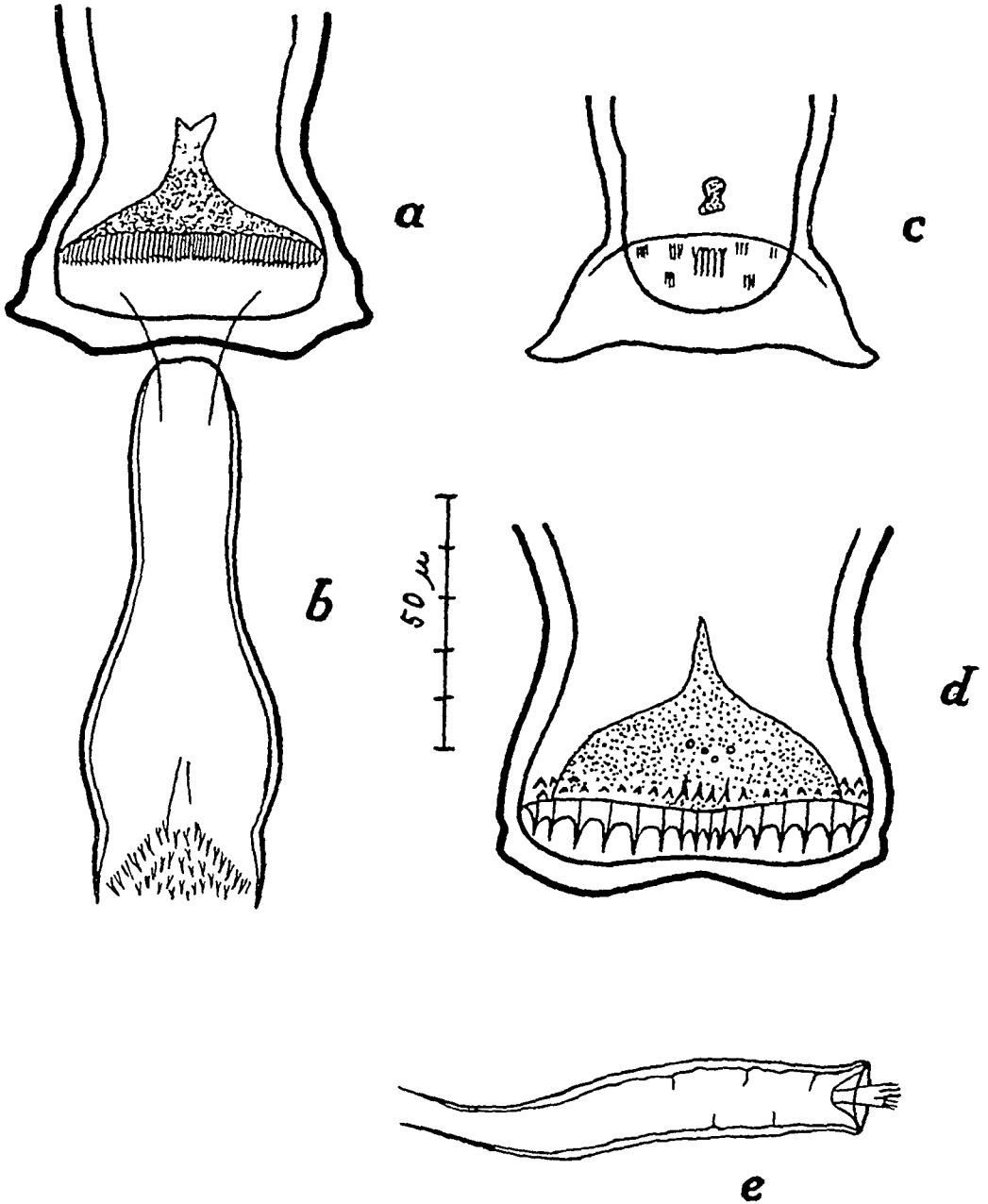
- (a) Buccal cavity of female of *P. zeylanicus*.
 (b) " " " " " *P. arboris*.
 (d) " " " male of *P. arboris*.
 (c) Pharynx of female of *P. arboris*.
 (e) Spermatheca of *P. arboris*.
 (f) " " *P. zeylanicus*.





- (a) Buccal cavity of female of *P. babu* var. *insularis* var. nov.
 (c) " " " " " *P. babu* (specimen from the Punjab).
 (b) Pharynx of female of *P. babu* var. *insularis*.
 (d) " " " " " *P. babu* (specimen from the Punjab).
 (f) " " " " " *P. babu* from Mauritius.
 (e) Spermatheca of *P. babu* var. *insularis*.

PLATE VIII.



- (a) Buccal cavity of female of *P. barraudi* (?) var. nov.
 (c) " " " " " *P. bailyi* var. *campester*.
 (b) Pharynx of female of *P. barraudi* (?) var. nov.
 (d) Buccal cavity of female of *P. iyengari* var. *malayensis* var. nov.
 (e) Spermatheca of female of *P. iyengari* var. *malayensis* var. nov.

P. stantoni.

The two specimens from Ceylon correspond in every detail to the description of Sinton, and of Raynal and Gaschen from Siam and Indo-China.

Material examined: 1 ♀, Kalagoda (Ceylon), 6-3-1934. 1 ♀, Katawa Panni (Ceylon), Jan. 1934 (Carter).

P. argentipes.

Material examined: 1 ♀, Godigomuwa, 13-11-1932. 2 ♂♂, 2 ♀♀, Pannipitiya, 5-2-1933. 1 ♀, Depanama Panni, 12-5-1934. 1 ♀, Katawa Panni, Jan. 1934. 1 ♂, 1 ♀, Kalagoda, 6-3-1934. Ceylon (Carter).

***P. squamipleuris* var. *indicus* Th.**

Material examined: 2 ♂♂, 2 ♀♀, Bangkok (Siam), July 1932 (Causey).

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THE ACTION OF CERTAIN CARDIAC DRUGS ON EMBRYONIC HEART EXPLANTS.

BY

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CARDIAC stimulants are often exhibited to revive a failing heart and it is a matter of some importance both from pharmacological and therapeutical viewpoints to know whether these remedies exert their action directly on the musculature of the heart or indirectly through the nervous mechanisms controlling the heart. Pharmacologists and physiologists have attempted to analyse and locate the seat of action of these drugs by a variety of experimental methods involving the use of 'denervated' or isolated hearts or by utilizing the selective affinity of certain chemical agents to produce depression or paralysis of ganglion cells and nerve-endings. The dual nature of the nerve supply and other complexities associated with cardiac enervation, however, offer considerable difficulties in the way of investigation and most of the data in this field are largely inferential and tentative. Thus, it is almost impossible to denervate completely the heart of adult vertebrates to test the action of drugs on the muscle. If the cervical and thoracic sympathetic chains are removed as has been done by Cannon *et al.* (1929), the sympathetic nerves of the heart degenerate completely and no regeneration is possible as the cell bodies have been removed. Cardiac vagus nerve, unlike the sympathetic, cannot, however, be entirely eliminated, as even after complete section of the vagus, the post-ganglionic fibres have been known to survive (Cameron, 1933) and, therefore, one can draw no safe conclusions regarding the site of action of drugs by

using the so-called 'denervated' hearts. By using an isolated heart, one may be sure that the effects on such a preparation must be of peripheral origin but this does not exclude the possibility that the action may be either through the intrinsic nerve endings or the neuromuscular substance of the heart or directly on the cardiac muscle. By the judicious use of chemical agents like nicotine, atropine and ergotoxine, very useful information regarding the point of attack of different cardiac remedies may be obtained but even these data are not always conclusive.

The only vertebrate hearts which have no enervation, neither sympathetic nor parasympathetic, are those of vertebrate embryos in the earlier stages of development. Shortly after the onset of heart function, regular rhythm and conduction develop even before the nerves reach the heart. The aneural embryonic heart, therefore, is the most satisfactory material in which to study the direct action of drugs on the muscle of the heart. The present study with fragments of such embryonic cardiac tissue cultivated *in vitro* was undertaken primarily to answer the fundamental question as to whether the commonly used cardiac 'tonics' exert a direct effect on the heart muscle and, if so, the nature and intensity of this action. Incidentally, it was expected that an investigation on these lines might throw some light on the neuro-humoral theory of Loewi and Dale which postulates that autonomic nervous impulses to the heart are mediated through the liberation of chemical agents of the type of adrenaline and acetylcholine.

MATERIALS AND METHODS.

Armstrong (1935) has shown that the hearts of the embryos of *Fundulus heteroclitus* and *Fundulus majalis* (salt-water minnows) are morphologically and physiologically aneural from the onset of heart beat after 72 hours of development until the 8th day. Markowitch (1931) working on the response of acetylcholine on chick-embryo hearts found evidence to indicate that such hearts were nerve-free up to 7 to 8 days. In our experiments, therefore, we thought it desirable to use only fragments of cardiac tissue from chick embryos between 2 and 7 days old.

The technique of tissue culture originally developed by Carrel (1912) and as modified in this Laboratory by Chopra, Das and Mukerji (1936) was employed. In brief, this was as follows: The hearts were removed aseptically from embryonic chicks of various ages (2 to 7 days) and were cut into fragments of about 1 sq. mm. in area. It was usually possible to do this with the naked eye; a dissecting microscope was seldom used in our series, as very small bits of heart tissue which were cultivated never survived for a sufficiently long time. The heart fragments were then suspended in a mixture of extracts derived from the embryo and of the homologous plasma obtained from a cock. Two sets of slides were prepared with a view to watch the two different characteristics of the heart explants at their best, namely, (1) the throbbing of the heart and (2) the fibroblastic growth of the tissue cells. In the first set of slides where the rate of throbbing was the important result to be observed, embryo extract was avoided as far as possible. However, a drop of extract was necessary to help in the clotting of the hanging drop. Under such conditions, the throbbing went on for a number of days (7 to 12 days) without change of medium. In the second set of slides where the idea was to watch the rate of tissue growth only, the explants were prepared with liberal quantities of embryonic extracts which apparently possessed a stimulating effect on tissue growth.

To study the effect of cardiac drugs, both types of explants were treated with various dilutions of the drugs under investigations and controls were kept side by side for purposes of comparison. More than a dozen explants were made in each case and every dilution was repeated at least five times. The slides were kept in an incubator at 37.5°C., and examined every day inside a microscope incubator to record the rate of throbbing. The relative growth of tissues in the second series of slides was also measured daily with the help of camera lucida on graph paper. The number of squares covered by a particular explant was calculated and the percentage increment from day to day was recorded and comparisons made with the controls of the same series. Usually, records were maintained for 8 to 9 days but, in a few cases, longer periods of observation were necessary.

The results obtained with lanadigin (pure glucoside of *D. lanata*), thevetin (pure glucoside from *T. nerifolia*), strophanthin (pure glucoside of *K. strophanthus*), caffeine (trimethylxanthine), and cardiazol (1,3,5-trimethyl-2-tetrazol) are presented in this paper. Pure lanadigin was obtained free of charge by Messrs. Beiersdorf, Ltd., the original manufacturers. Pure thevetin (M. P. about 192°C.) was isolated from the original plant in the Department of Chemistry, School of Tropical Medicine. International standard strophanthin was obtained from the National Institute for Medical Research, London. Pure caffeine (Merck) and cardiazol (Knoll) were procured from the market. Solutions were prepared fresh on the days that tissue cultures were put up. A stock solution of 1 : 1,000 was prepared for each drug and it was passed through L-3 candles to render them bacteria-free. These stock solutions were subsequently diluted with Pannet and Compton's (1924) isotonic solution for use in the tissue explants.

RESULTS.

The most significant results on the rate of throbbing (after 48 hours' incubation) as well as on the extent of tissue proliferation (after 60 hours' incubation) are shown in the Table and are also graphically represented (Graphs 1 and 2). These results, however, are generally representative of the type of changes occurring in the explants during the whole period that it was possible to keep them alive. It will be seen from a careful perusal of the Table that the first three drugs, namely lanadigin, thevetin, and strophanthin, have produced qualitatively similar reactions, although there are distinct differences in the degree of response in each case. In dilutions of 1 in 10,000 to 1 in 50,000, lanadigin brings about a definite inhibition in the rate of pulsations. The rate of fibroblastic outgrowth of the explants is also markedly retarded in these concentrations. As the strength of the drug is increased from 1 in 1,000 to 1 in 100, the inhibitory effect is changed gradually into a toxic effect, as is evidenced by a complete stoppage of the pulsations and ultimately to a fatty infiltration of the cells and complete disorganization of the tissue fragments. If the explants are exposed to a dilution weaker than 1 in 50,000, the inhibitory action is only feebly marked. The rate of throbbing is diminished, sometimes from the normal average of 75 beats per minute to about 40 beats per minute, but the pulsations are seen to become more powerful and more prolonged. The interval between any two beats appears to become longer, though for want of a suitable method to measure this phase, it is not possible for us to make a

TABLE.

Effect of cardiac drugs on heart explants.

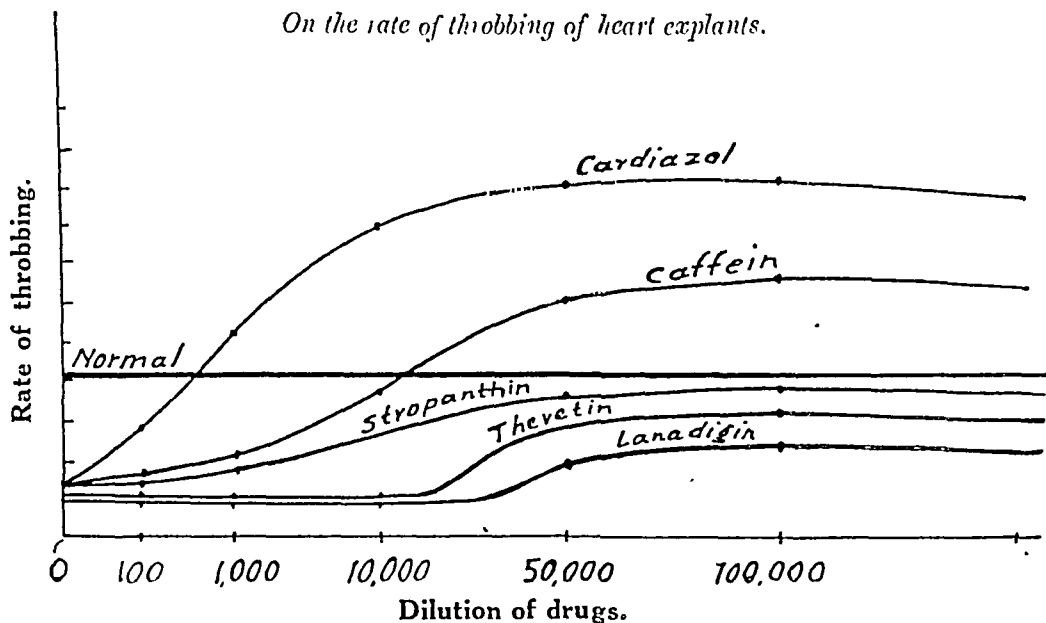
Dilution.	ON THE RATE OF THROBBING (AFTER 48 HOURS).						ON THE TISSUE GROWTH (AFTER 60 HOURS).					
	100	1,000	10,000	50,000	100,000	500,000	100	1,000	10,000	50,000	100,000	500,000
Lanadigin ..	Stop	Stop	Stop	Slow	Slow	Slow	Nil	Nil	Nil	Less than control —	Same as control ±	Enhanced +
Thevetin ..	Stop	Stop	Stop	Slow	Slow	Slow	Nil	Nil	Less than control —	Less than control —	Enhanced +	Enhanced ++
Strophanthin	Stop	Stop	Slow	Slightly enhanced followed by slowing.	Slow	Slow	Nil	Nil	Less than control —	Same as control ±	Enhanced ++	Enhanced +++
Caffeine ..	Stop	Slow ; enhanced later.	Enhanced	Enhanced	Enhanced	Enhanced	Nil	Less than control —	Less than control —	Enhanced +	Enhanced ++	Enhanced +++
Cardiazol ..	Slow ; enhanced later.	Enhanced slightly.	Enhanced	Enhanced	Enhanced	Enhanced	Slightly less than control ±	Less than control —	Enhanced +	Enhanced ++	Enhanced ++	Enhanced +++

N.B.—

+ means approximately 25 per cent increment of fibroblastic out-growth compared to normal controls.
 ++ means 50 per cent.
 +++ means 75 per cent and so on.
 ± indicates doubtful increase.
 — indicates negative increase (less than control).

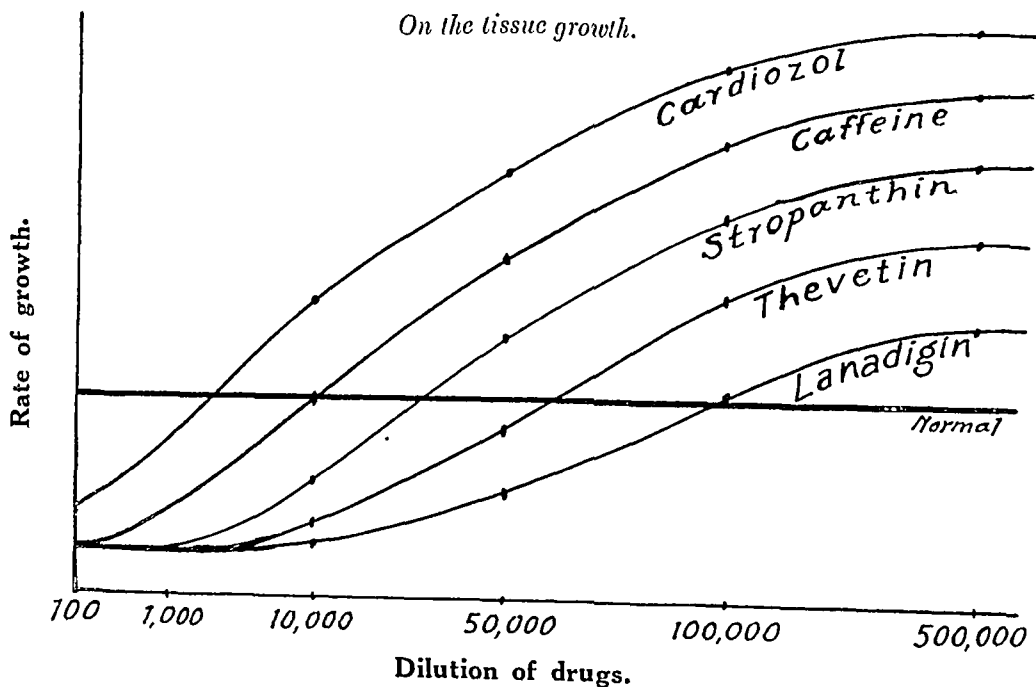
GRAPH 1.

On the rate of throbbing of heart explants.



GRAPH 2.

On the tissue growth.



definite statement. The tissue proliferation also is stimulated in these concentrations, being more definitely noticeable in a dilution of 1 in 100,000 than in 1 in 50,000. Thevetin and strophanthin are slightly weaker than lanadigin, the inhibitory effect on the rate of throbbing in both the cases disappearing in a dilution in the neighbourhood of 1 in 10,000. Strophanthin in weaker dilutions has sometimes caused an initial enhancement of the rate of pulsations which, however, is definitely slowed in the later stages. The tissue-growth retarding effects are also comparatively less than lanadigin (see Plate IX, figs. 1, 2, 3, 4, 5, 6, and 7). Caffeine and cardiazol have produced effects on the explants which are sharply in contrast to those produced by the first three drugs. Except in a dilution of 1 in 100, both these drugs have manifested a markedly stimulant action; the rate of pulsations shows a tendency to increase from the very onset and they become stronger and more forceful. The fibroblastic out-growth proceeds at a rapid pace and almost fills up the whole slide within a comparatively shorter period (see Plate IX, figs. 8 and 9). The explants can be kept alive for a much longer period and the throbbing goes on with uninterrupted vigour. The cells which proliferate are healthy in appearance and do not tend to show fatty infiltration as is commonly seen with the lanadigin explants in the later stages (9 to 10 days). Cardiazol appears to be slightly more powerful than caffeine, though the latter apparently possesses a peculiar property of maintaining a tissue explant throbbing for a longer period of time.

DISCUSSION.

From the results obtained, certain interesting conclusions may be drawn. It seems clear that the three glucosides tested in our series possess a predominantly inhibitory effect on cardiac explants, whereas both caffeine and cardiazol exert a predominantly stimulant and tonic effect. It is well known that the pharmacological actions of digitalis and strophanthin are very similar and Chopra and Mukerji (1933) and, later, Chen and Chen (1934) have shown that the glucoside of *T. nerifolia* exert a digitalis-like action on the heart. The qualitatively identical nature of response on cardiac explants goes to show that not only the nature of action but the mechanism and site of action of the three glucosides may also be the same. It has been often contended, sometimes on excellent pharmacological evidence, that digitalis is chiefly a vagomimetic drug and the therapeutic effects observed in auricular fibrillation and in other types of loss of compensation are due to central and peripheral vagal stimulation diminishing cardiac tone, excitability and rate of conduction through the Purkinje system. While this may be true in the intact mammalian heart, experiments on isolated strips of cardiac tissue or on isolated perfused hearts show quite a noticeable preponderance of muscular effects over vagal effects, and indicate that digitalis-like drugs may have some direct muscular action in addition to the vagal action. The question now arises as to whether the effect on the muscle is one of stimulation or depression. The experimental evidence on this point is contradictory. That digitalis bodies may possess an inherent stimulant effect on auricular and ventricular muscle is seen by its power to increase the speed of conduction in the auricles and also the rate of fibrillation in cases of auricular and ventricular fibrillation. Working with frog-heart preparations, Loewe (1918) observed increased systolic excursions and lengthened diastolic pauses. Sasaki (1921) also found evidence of increased

PLATE IX.

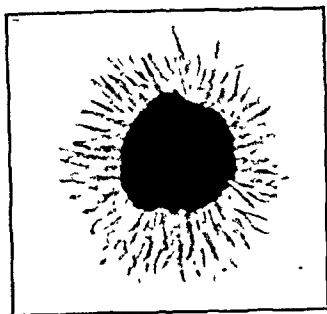


FIG. 1.

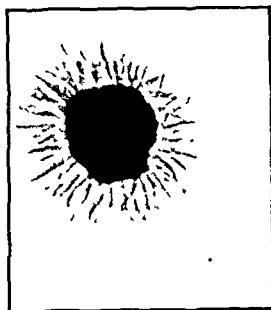


FIG. 2.

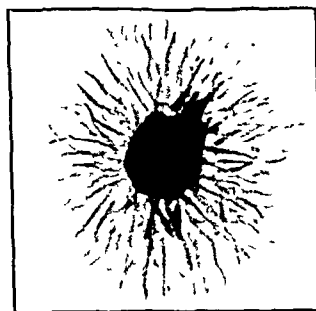


FIG. 3.

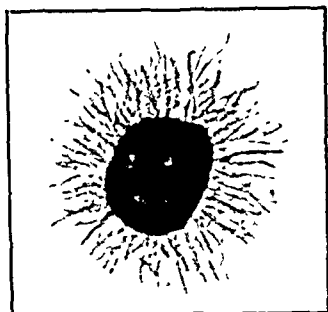


FIG. 4.

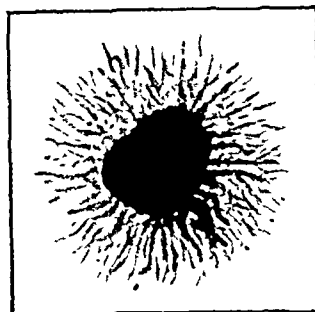


FIG. 5.

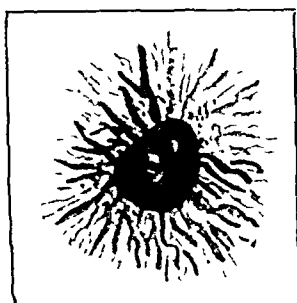


FIG. 6.

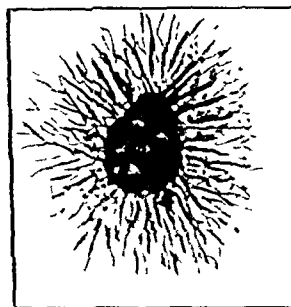


FIG. 7.

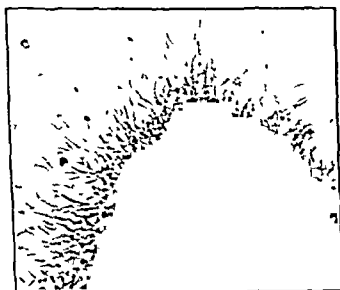


FIG. 8.



FIG. 9.

Figs. 1, 2, 3, 4, 5, 6, and 7.—Camera-lucida drawings of cardiac explants.

Fig. 1.—Control explant. Fig. 2.—Lanadigin (1 in 30,000) showing retardation of growth. Fig. 3.—Lanadigin (1 in 100,000) showing slightly improved growth than control. Fig. 4.—Thevetin (1 in 100,000) slight stimulation of growth. Fig. 5.—Strophanthin (1 in 100,000) slightly better than thevetin. Figs. 6 and 7.—Caffeine and cardiazol (1 in 10,000 and 1 in 5,000 respectively). Figs. 8 and 9.—Photomicrograph from slides showing extensive tissue out-growth in Fig. 9 which has been treated with caffeine. Fig. 8 is the corresponding control.

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STUDIES ON SOME DEXTRO-ROTATORY HYDROCUPREIDINE DERIVATIVES.

Part I.

COMPARATIVE HÆMOLYTIC ACTIVITY.

BY

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THE alkyl ethers of hydrocupreines have been synthesized in pure form by Giemsa and Halberkann (1918) and also by Heidelberger and Jacobs (1922). Morgenroth *et al.* (1922-1926) and Giemsa *et al.* (1917-1926) have carried out extensive studies on their pharmacological and therapeutic properties particularly with reference to their local anæsthetic properties, antimalarial activities, antiseptic properties in wound infections, and bactericidal properties in systemic infections caused by such organisms as streptococcus and pneumococcus. The hydrocupreidine derivatives have apparently up till now not been pharmacologically examined. Acton (1921), Chopra (1922), and later Acton and Chopra (unpublished) carried out some studies on ethyl hydrocupreidine which indicated that the dextro-rotatory derivatives might be more potent therapeutic agents than their levo-rotatory isomers. This led to the synthesis of the following compounds at the School of Tropical Medicine by Ghosh and Chatterjee (1931; 1932) and a systematic investigation has now been undertaken.

Table I gives the details of the compounds prepared:—

TABLE I.
Hydrocupreidine derivatives.

Number.	Name of base and empirical formula.	Structural formula.*	Melting point, C.	Solubility.	Optical rotation.	Crystalline structure.
1	Hydrocupreidine $C_{10}H_{24}O_2N_2$	R.OH	195°	Alcohol ether $CHCl_3$ benzene.	+ 253.4° (C = 1.42 in alcohol).	Cream-tinted hexagonal plates.
2	Methylhydrocupreidine (Hydroquinidine) $C_{20}H_{26}O_2N_2$	R.OCH ₃	166°–167°	do.	+ 230°	Long needles.
3	Ethylhydrocupreidine $C_{22}H_{28}O_2N_2$	R.OC ₂ H ₅	197.5°–198°	do.	+ 212.8° (C = 1.008 in alcohol).	Slender needles.
4	n-propylhydrocupreidine $C_{22}H_{30}O_2N_2$	R.OC ₃ H ₇	182°	do.	+ 206.25° (C = 1 in $CHCl_3$).	Long needles.
5	iso-propylhydrocupreidine $C_{32}H_{30}O_2N_2$	R.OC ₃ H ₇	181°	do.	+ 206.25° (C = 1 in $CHCl_3$).	Colourless plates.
6	n-butylhydrocupreidine $C_{24}H_{32}O_2N_2$	R.OC ₄ H ₉	176°	do.	+ 194.5° (C = 1 in $CHCl_3$).	Needles.
7	Iso-butylhydrocupreidine $C_{23}H_{32}O_2N_2$	R.OC ₄ H ₉	175°	do.	+ 186.7° (C = 2 in $CHCl_3$).	Needles.

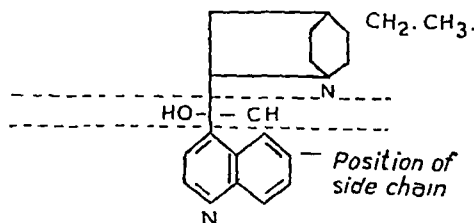
* See opposite page for reference.

TABLE I—concl.

Number.	Name of base and empirical formula.	Structural formula.*	Melting point, C.	Solubility.	Optical rotation.	Crystalline structure.
8	n-amyl hydrocupreidine $C_{21}H_{35}O_2N_2$	$R.OC_5H_{11}$	161°	Alcohol ether $CHCl_3$ benzene.	+ 189.25° (C = 1 in chloroform).	Long needles.
9	Is o-amyl hydrocupreidine $C_{24}H_{39}O_2N_2$	$R.OC_5H_{11}$	168°	do.	+ 201° (C = 2 in abs. alcohol).	Microscopic needles.
10	n-hexyl hydrocupreidine $C_{23}H_{37}O_2N_2$	$R.OC_6H_{13}$	—	—	—	—
11	n-heptyl hydrocupreidine $C_{26}H_{41}O_2N_2$	$R.OC_7H_{15}$	158°	Alcohol ether $CHCl_3$ benzene.	+ 179.75° (C = 1 in chloroform).	Long silky needles.
12	n-octyl hydrocupreidine $C_{27}H_{43}O_2N_2$	$R.OC_8H_{17}$	151°	do.	+ 170.75° (C = 1 in chloroform).	Thin needles.
13	Sec.-octyl hydrocupreidine $C_{27}H_{43}O_2N_2$	$R.OC_8H_{17}$	—	—	—	—

The base could not be crystallized. The corresponding hydrochloride salts were, however, obtainable in pure form.

* 'R' stands for



The present investigation concerns itself with a study of the comparative hemolytic activity. The comparison has been made with quinine and also with the corresponding hydrocupreine derivatives which are available.

METHODS.

Red blood corpuscles.—Red blood corpuscles of rabbits and human beings were used for this study. The blood was obtained from the ear-vein of rabbits, defibrinated, and the corpuscles separated from the plasma by centrifugalization. Venous blood from healthy laboratory assistants and apparently healthy patients attending the skin clinic of the School of Tropical Medicine, was collected in potassium oxalate tubes and centrifugalized.

The corpuscles were then washed and centrifugalized three times with physiological saline. This treatment was usually sufficient to render the supernatant fluid colourless. Where even a slight colouring of the supernatant fluid was discovered, an additional washing was resorted to before the corpuscles were used for studies on hæmolysis.

Preparation of solution.—As the hydrocupreidine bases are only soluble in organic solvents, the corresponding hydrochloride salts were used for the experiments. Most of the salts are soluble enough to enable a 0.5 per cent solution to be easily prepared and provided the pH is properly adjusted, the solutions can be maintained without precipitation for a fairly long time. The higher homologues are, however, very sparingly soluble and only very weak solutions can be prepared. The octyl salt is often precipitated when dilutions were made with physiological saline. In these cases, dilutions were made with a solution of hydrochloric acid of pH 5.6.

Hydrogen-ion concentration.—Acton (*loc. cit.*) showed that quinine base had very slight hæmolytic property and that the hæmolysis reported after quinine administration was due primarily to acidity of the hydrochloride salt used in therapeutics rather than to the base itself. In these experiments, the hydrogen-ion concentration was, therefore, rigidly controlled and though it was not possible to bring the solutions near the neutral point on account of the sparing solubility of some of the higher homologues of the series, attempt was made to keep the pH at the range between 5.3 and 5.8 by the judicious addition of phosphate buffer. Controls with weak acid of the same pH range were run side by side whenever necessary. However, it was found that in case of quinine solutions, changes of pH between 5.3 and 5.8 had little effect in bringing about an acceleration of hæmolysis.

Hæmolysis studies.—As this was only a comparative study, no attempt was made to construct 'percentage hæmolysis curves' by calculating the percentage of cells hæmolysed in relation to the total number of corpuscles, as recommended by Ponder (1923) and Ponder and Yeager (1930). Two comparatively simple methods were devised to observe the hæmolytic reaction. In the first series, (1) *the minimum time for the starting of hæmolysis* when the corpuscle suspensions were kept in contact with various dilutions of the hydrocupreidine derivatives under investigation, were recorded. A series of dilutions ranging from 1 in 250 to 1 in 50,000 were employed in each case and the results obtained were used in plotting a 'time-dilution curve'. It is not always easy to detect the exact time at which hæmolysis just starts by simple observation of the translucency of the corpuscular suspensions in glass tubes. In cases of doubt, it was found convenient to hold the tubes against a powerful electric bulb in front of which a metal diaphragm with a slit-like aperture

was attached. As soon as the corpuscles began to get laked, the cell suspension became translucent at the top and the light spot was clearly visible for that area. In the second series, (2) *the minimum concentration just necessary to produce haemolysis* was observed by keeping the time of exposure of the corpuscular suspensions to the different drugs constant. Equal volumes of various dilutions of the particular drug to be tested were added to cell suspensions, the tubes were gently rotated to help uniform mixture, and after exactly 20 minutes, the tubes were centrifugalized and the evidence of haemolysis, if any, was looked for in the supernatant fluid. Free haemoglobin was easily detected from the pink colour of the supernatant fluid. Presence of broken corpuscles and corpuscular debris were also seen under the microscope. Controls without the addition of drugs were run side by side in every case to avoid any fallacy in the observations. The minimum concentration just necessary to produce haemolysis with quinine hydrochloride is given the arbitrary value of unity and the haemolytic potency of the other derivatives is expressed in terms of quinine.

RESULTS.

The experimental findings are recorded in Tables II and III and are also graphically represented (Graphs 1 and 2). The effect of the hydrocupreidine derivatives only are exhibited; the levo-rotatory compounds behave in practically the same way and the differences in haemolytic potency of the members available for study are so insignificant that a separate table is not necessary. Washed human red blood corpuscles were generally employed. The results with the rabbit corpuscles were practically identical, and hence are not separately mentioned.

A consideration of Table II and Graph 1 shows clearly that the hydrocupreidine derivatives are very powerful haemolytic agents, the activity increasing steadily with the increase in the number of carbon atoms in the side chain. The higher is the drug in this series, the quicker is the onset of haemolysis and the reaction becomes also completed within a shorter period. The rapidity with which the reaction takes place in some cases indicates that haemolysis with these derivatives is probably of the type of 'saponin' haemolysis and not 'osmotic' haemolysis. The increase of haemolytic activity with the increase in the complexity of the side chain is also borne out from a consideration of Table III and Graph 2. Taking quinine as unity, the numerical representation of the activity of the octyl salts are about 120. An interesting point is the behaviour of the iso-butyl salt which appears to be nearly 10 times stronger than its homologue. In the case of the amyl and propyl derivatives, however, there is no evidence that the iso-compounds are any stronger than the normal derivatives. This point is being more fully investigated.

The intense haemolytic activities manifested by the last 3 or 4 members of the series (n-hexyl, n-heptyl, n-octyl, sec.-octyl) would ordinarily preclude the possibility of their being used in systemic medication even if later investigations prove them to be powerful pharmacodynamic and therapeutic agents. The effect of the addition of serum on the cupreidine corpuscle haemolytic system was, therefore, also investigated. It will be seen from Table III that such addition of serum has an appreciable effect on the haemolytic process. The higher homologues show greater response as far as the

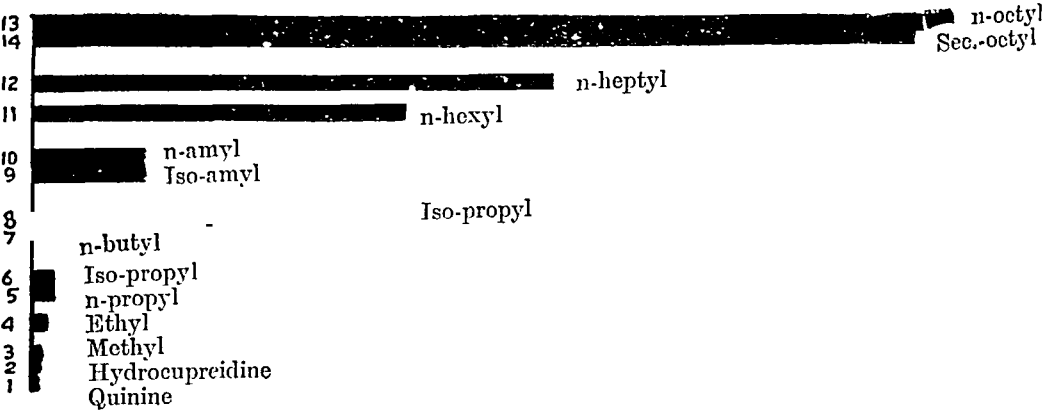
TABLE III.

Relative hæmolytic potency of hydrocupreidine derivatives.

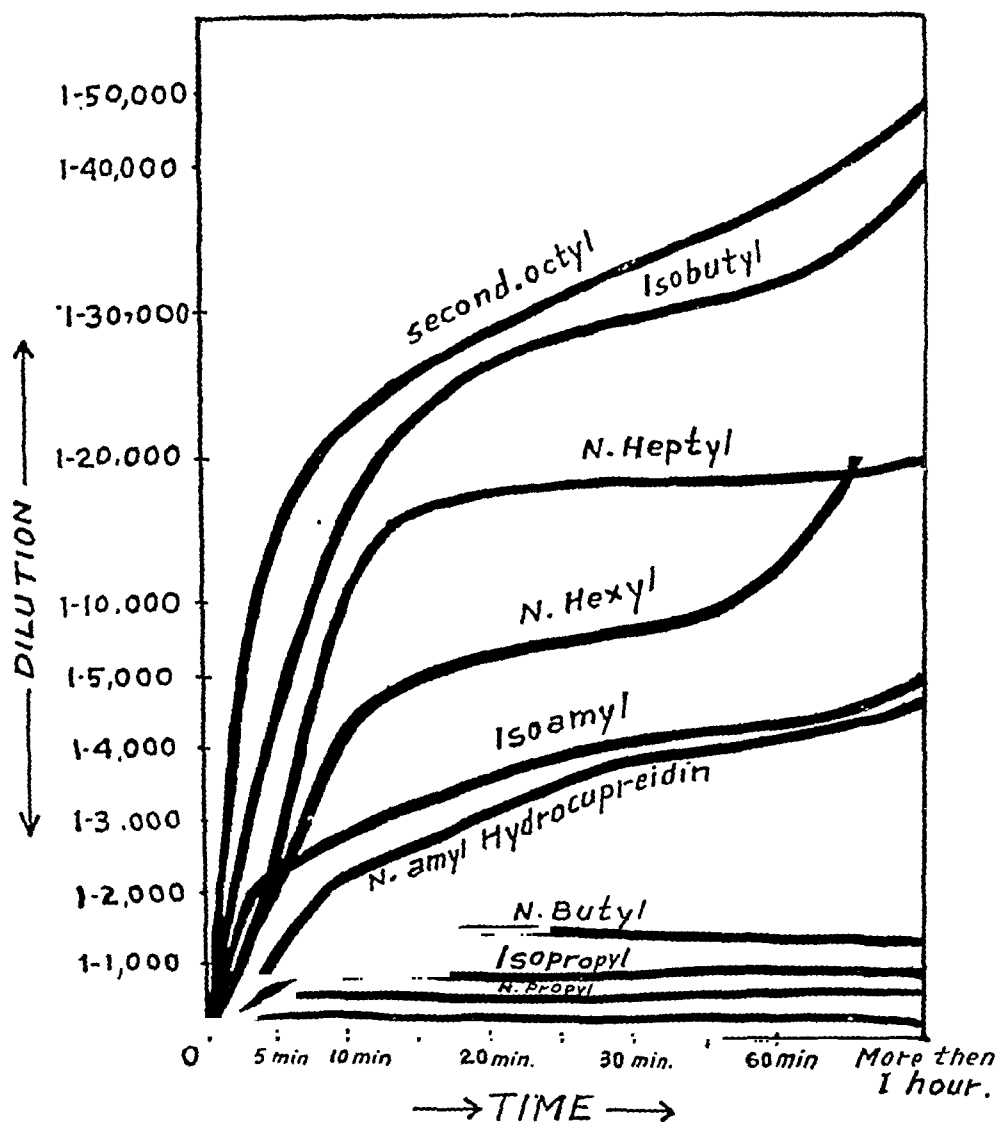
Name of compounds.	Minimum concentration for hæmolysis.	Minimum concentration for hæmolysis with serum.	Relative hæmolytic potency. (Quinine = 1)
Quinine HC.	1-400	1-250	1·0
Hydrocupreidine HCl. ..	1-500	1-400	1·25
Hydroquinidine HCl. (Methyl hydrocupreidine HCl) ..	1-500	1-400	1·25
Ethyl	1-800	1-400	2·0
n-propyl	1-1,000	1-400	2·5
Iso-propyl	1-900	1-500	2·3
n-butyl	1-1,800	1-800	4·75
Iso-butyl	1-18,000	1-8,000	47
n-amyl	1-5,000	1-3,000	15
Iso-amyl	1-5,000	1-3,000	15
n-hexyl	1-20,000	1-8,000	50
n-heptyl	1-28,000	1-9,000	70
n-octyl	1-50,000	1-28,000	125
Secondary-octyl	1-48,000	1-25,000	120

GRAPH 1.

Relative hæmolytic potency



GRAPH 2.

*Rate of Haemolysis during the 1st hour*

inhibition of haemolysis is concerned. The heptyl and octyl salts are, however, still highly haemolytic and this fact needs careful consideration in connection with their use in practical therapeutics. The mechanism by which such inhibition of haemolysis is brought about was not investigated. The alkaloids probably react with the protein component of the plasma to form non-lytic compounds. Similar

phenomena are known to occur in saponin or bile-salt hæmolytic systems where the addition of serum or plasma causes a definite inhibition of the hæmolytic reaction.

CONCLUSIONS.

1. The hæmolytic activity of hydrocupreidine derivatives increases in potency with the increase in the number of carbon atoms in the side chain. The hæmolysis tends to be more complete with the higher homologues than with the lower ones where partial hæmolysis is more common. An exception is noted in the case of iso-butyl salt for which no explanation is at present forthcoming.

2. The iso-compounds are practically identical in potency with the normal derivatives. The iso-propyl derivative is probably slightly weaker in action.

3. Hydrocupreine and hydrocupreidine derivatives are similar as far as their hæmolytic activity is concerned.

4. The hæmolytic potency diminishes greatly in presence of serum or plasma. In this respect, the members higher up in the series show greater response than the lower homologues.

5. The hydrogen-ion concentration has an appreciable influence of the hæmolytic process and the phenomena resemble more 'saponin' hæmolysis than 'osmotic' hæmolysis.

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STUDIES ON SOME DEXTRO-ROTATORY HYDROCUPREIDINE DERIVATIVES.

Part II.

COMPARATIVE ACTION ON DIGESTIVE ENZYMES.

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SOMORDINTZEV *et al.* (1927 to 1929) have made a comprehensive study of the action of various concentrations of quinine on the different digestive enzymes, and their studies have thrown considerable light on various aspects of the problem. Contrary to the general idea that cinchona alkaloids always inhibit the digestive enzymes, they discovered that therapeutic doses of quinine hydrochloride did not disturb the progress of peptic digestion in the stomach in the presence of normal acidity. In connection with the investigation of the pharmacological action and therapeutic properties of the hydrocupreidine derivatives, it was considered of interest to study the comparative action of these derivatives with their corresponding hydrocupreine salts and quinine hydrochloride on digestive enzymes. The effects on the salivary, pancreatic, and intestinal enzymes responsible for carbohydrate digestion (amylases and invertases) are presented in this paper.

METHODS.

The flow of saliva was stimulated by chewing a bit of paraffin wax and a quantity of saliva was collected in a beaker; 0.5 c.c. of the filtered saliva was then made up to 50 c.c. The amylase content of saliva in this dilution was found

TABLE I.

Levo-rotatory compounds.	SALIVARY AMYLASE.		PANCREATIC AMYLASE.		INVERTASE.	
	Activity expressed as c.c. of N/10 $\text{Na}_2\text{S}_2\text{O}_3$.	Percentage of inhibition.	Activity expressed as c.c. of N/10 $\text{Na}_2\text{S}_2\text{O}_3$.	Percentage of inhibition.	Activity expressed as c.c. of N/10 $\text{Na}_2\text{S}_2\text{O}_3$.	Percentage of inhibition.
Original activity	5.0	..	4.0	..	5.0	..
Quinine HCl ..	4.25	15	3.4	15	4.70	6
Hydroquinine HCl.	4.55	9	3.6	10	4.70	6
Ethyl hydrocupreine HCl.	4.40	12	3.50	12.5	4.70	6
Iso-butyl hydr. HCl.	3.95	21	3.10	22.5	3.70	26
Iso-amyl hydr. HCl.	3.00	40	2.30	42.5	3.50	30
Iso-heptyl hydr. HCl.	1.50	70	1.15	71.25	4.40	12
Iso-octyl hydr. HCl.	1.50	70	1.15	71.25	4.80	4

TABLE II.

Dextro-rotatory compounds.	SALIVARY AMYLASE.		PANCREATIC AMYLASE.		INVERTASE.	
	Activity expressed as c.c. of N/10 $\text{Na}_2\text{S}_2\text{O}_3$.	Percentage of inhibition.	Activity expressed as c.c. of N/10 $\text{Na}_2\text{S}_2\text{O}_3$.	Percentage of inhibition.	Activity expressed as c.c. of N/10 $\text{Na}_2\text{S}_2\text{O}_3$.	Percentage of inhibition.
Original enzyme activity.	5.0	..	4.0	..	5.0	..
Quinine HCl ..	4.25	15	3.4	15	4.7	6.1
Hydrocupreidine HCl.	5.0	Nil	4.05	Nil	5.0	Nil.
Methyl hydr. HCl.	5.0	Nil	4.05	Nil	5.0	Nil.
Ethyl hydr. HCl	5.0	Nil	4.00	Nil	5.0	Nil.
n-propyl hydr. HCl.	4.8	4	4.00	Nil	5.0	Nil.

TABLE II—*concl'd.*

Dextro-rotatory compounds.	SALIVARY AMYLASE.		PANCREATIC AMYLASE.		INVERTASE.	
	Activity expressed as c.c. of N/10 $\text{Na}_2\text{S}_2\text{O}_3$.	Percentage of inhibition.	Activity expressed as c.c. of N/10 $\text{Na}_2\text{S}_2\text{O}_3$.	Percentage of inhibition.	Activity expressed as c.c. of N/10 $\text{Na}_2\text{S}_2\text{O}_3$.	Percentage of inhibition.
Iso-propyl hydr. HCl.	4.8	4	3.95	Nil	5.0	Nil.
n-butyl hydr. HCl.	4.8	4	3.90	2.5	4.80	4
Iso-butyl hydr. HCl.	4.8	4	3.85	3.8	4.80	4
n-amyl hydr. HCl.	4.65	7	3.80	5	4.5	10
Iso-amyl hydr. HCl.	4.65	7	3.80	5	4.5	10
n-hexyl hydr. HCl.	4.45	11	3.60	10	4.5	10
n-heptyl hydr. HCl.	4.45	11	3.60	10	4.4	12
Sec.-octyl hydr. HCl.	4.35	13	3.50	12.5	4.4	12
n-octyl hydr. HCl.	4.35	13	3.50	12.5	4.4	12

An examination of the above tables reveals that the levo-rotatory hydrocupreines are far more powerful inhibitors to the enzymes than the dextro-rotatory hydrocupreidines. Acton (1921), however, came to the conclusion that the dextro-rotatory cinchona alkaloids were more powerful in their inhibitory actions on enzymes than the levo-rotatory isomers. This might be due to the fact that the more accurate and modern quantitative methods for studying enzymatic activity were not available in those days. The higher members of the levo-rotatory hydrocupreine series exert stronger inhibitory action on the amylases than on the invertases, whereas the dextro-rotatory derivatives are almost similar in their behaviour towards all the three enzymes. Judging from their inhibitory activity on invertase, iso-butyl and iso-amyl hydrocupreines appear to be the most potent of the series.

The addition of each CH_2 group to the basic molecule of the compounds under investigation retards the amylolytic digestion considerably, indicating interesting possibilities with reference to the character of the active enzyme grouping. In the case of hydroquinine (methyl hydrocupreine), the percentage of inhibition is only 9 as against 70 with iso-octyl hydrocupreine. It is possible that these alkyl

radicals react with the active enzyme group resulting in varying degrees of inactivation depending upon the number of CH_2 groups. The depression of activity in the case of invertase apparently does not bear any relationship with the addition of CH_2 groups to the molecule. This suggests that the mode of inactivation of the invertase is probably different from that of the amylases.

SUMMARY AND CONCLUSIONS.

1. The comparative inhibitory action of a series of dextro-rotatory hydrocupreidine derivatives and their corresponding levo-rotatory isomers, wherever available, on salivary and pancreatic amylases and on yeast invertase was studied. Quinine was taken as the standard of comparison.

2. The levo-rotatory hydrocupreines are found to be far more powerful inhibitors to the amylases than the dextro-rotatory hydrocupreidines. Quinine comes in between the two groups, being generally weaker than the higher members of the levo-rotatory hydrocupreines but stronger than the dextro-rotatory hydrocupreidines. The lower members of the hydrocupreidine series do not appear to possess any inhibitory action at all on invertase activity, while the higher homologues (from butyl-derivative upwards) are definitely more potent in this respect. The mechanism of inactivation has not been worked out in detail but the evidence available seems to indicate that the alkyl radicals (CH_2 groups) react with active enzyme groups of the amylases and that the mode of inactivation of invertase is probably different from that of the amylases.

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THE EFFECTS OF ANTERIOR PITUITARY EXTRACTS AND CHOLINE ON THE LIVER-FAT OF RABBITS.

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THE excessive increase in the neutral fat of the liver ('fatty infiltration') and the change in the physical characteristics of the fat with more or less extensive damage to the liver cells ('fatty degeneration') are commonly met with in a variety of physiological and pathological conditions. In the early stages of starvation, for example, fatty changes in the liver are commonly seen and a fat-rich diet is frequently accompanied with fatty livers. Several substances like cholesterol, alcohol, phloridzin, chloroform, phosphorus, and carbon tetra-chloride are known to produce fatty livers and the association of diseases like pernicious anæmia, yellow fever, acute yellow atrophy, eclampsia, etc., with fatty degeneration of the liver is already well established. The mechanism of production of these changes, however, is still obscure. During recent years, a number of interesting observations on the influence of pituitary extracts and choline on the fat metabolism of the liver have been published which promise to have an important bearing on many aspects of this question.

Coope and Chamberlain (1925) showed that the injection of posterior-lobe pituitary extract was followed by an increase in the concentration of the liver-fat. Oshima (1929), Hynd and Rotter (1932), White (1933) and Mukerji and van Dyke (1935) confirmed and extended the experiments of Coope and Chamberlain. That the anterior pituitary extract also has some effect on the metabolism of fats was first pointed out by Burn and Ling (1930) who noted increased excretion of ketone bodies following injection of anterior pituitary in rats fed on a fat diet. Anselmino and Hoffmann (1931) found an increase in the ketone content of rats' blood and Magistris (1932) reported similar findings in the rabbit. Best and Campbell (1936)

have conclusively demonstrated extensive deposition of liver-fat in rats after the injection of anterior pituitary extracts.

During the course of an extensive investigation on the mechanism of fatty infiltration of the liver in de-pancreatized dogs, Best and Huntsman (1932) discovered that the feeding of choline effectively prevented the deposition of liver-fat in such animals. This interesting observation has since been extended and it has been shown that choline cures fatty livers due to starvation, a fat-rich diet or a diet rich in cholesterol (Best and Ridout, 1933; Best, Hershey and Huntsman, 1932; Best, Channon and Ridout, 1934). Whether choline would be able to exert a similar effect and antagonize the effect of the pituitary extracts on the 'liver-fat' is a pertinent question. Mukerji and van Dyke (*loc. cit.*) showed that the feeding of choline chloride had no effect in preventing the fatty infiltration due to injection of posterior pituitary extracts. Data with regard to the effect of the anterior pituitary were not available at that time, and the investigation could not be pursued further. By means of the experiments reported in this paper, it is hoped to throw further light on this interesting problem.

METHODS.

A group of 24 young healthy rabbits weighing on an average between 1 kg. and 1.5 kg. were chosen from a stock colony, were divided into three groups of eight each and were kept under identical conditions in a well-ventilated room on an unrestricted diet of green grass, soaked peas, fresh cabbage or lettuce and carrots. A week was allowed for the rabbits to get acclimatized to the laboratory conditions before the first series of experiments were started. Most of the animals gained weight during the period of observation.

Anterior pituitary gland tablets were generously supplied by the Calcutta branch of Messrs. Parke, Davis & Co. A fresh supply of dried gland powder was also obtained from the British Drug Houses. The material administered to the rabbits was extracted from the crude anterior-lobe powder with 40 volumes of N/25 HCl according to the method outlined by Best and Campbell (*loc. cit.*). A bulky protein precipitate was removed from this extract by adjusting to pH 5.2 with NaOH. One volume of 95 per cent alcohol was added to the filtrate and the reaction was adjusted to pH 5.2. The precipitate was removed and the filtrate evaporated *in vacuo*, at approximately 35°C. to 40°C., to a volume which corresponds to a 10 per cent solution of the original powder. A single large dose of anterior-lobe extract (400 mg.-kg.) was mixed with sterile gum arabic solution and injected subcutaneously about 24 hours before the animals were killed. Food was withheld during this period. The choline chloride was secured from Schering Kahlbaum & Co. In the form of a 10 per cent aqueous solution, it was administered once daily by means of a stomach tube. Enough water was employed to ensure the administration of the whole dose. The dosage employed was 500 mg.-kg., which was found by Best and Huntsman (*loc. cit.*) to be sufficient to prevent fatty infiltration of liver in the rats.

The concentration of fat in the liver was determined by a combination of the methods of Turner (1930) and of Leathes and Raper (1925). Turner's method was employed to remove the non-saponifiable matter immediately after saponification. The fatty acids were then determined by the method of Leathes and

Raper (*loc. cit.*). The iodine numbers of the fatty acids were estimated by the method of Rosenmund and Kuhnhehn (1923) which is now generally considered to yield less erroneous results than the time-honoured Wij's method. Triplicate samples of liver-tissue, mixed after being finely chopped, were used for the determination of liver fatty acids. Iodine numbers were determined in duplicate.

EXPERIMENTAL RESULTS AND COMMENT.

The most satisfactory experiments are summarized in Tables I, II, and III. The normal concentration of liver fatty acids was determined in the first group (Table I). The second group (Table II) was injected with anterior pituitary extract at least 21 hours before the determination of the liver fatty acids. The third group (Table III) was fed daily with choline chloride for eight to nine days and, at the expiry of this period, were injected with anterior pituitary extract and killed the following day for the estimation of liver fatty acids.

TABLE I.

The concentration of fat in the liver of the rabbit.

Serial number.	Sex.	Weight, kg.	LIVER.		
			Weight, g.	FATTY ACIDS.	
				Concentrations, per cent.	Iodine numbers.
1	Male ..	0.82	33	3.36	105
2	Female ..	1.00	52	3.54	113
3	Male ..	1.54	54	3.00	114
4	Female ..	1.12	39	3.25	129
5	Female ..	1.22	51	3.07	121
6	Male ..	1.12	58	2.75	114
7	Female ..	1.31	55	3.42	115
8	Male ..	1.20	64	2.72	130

- TABLE IV.

Comparison of averages from Tables I, II, and III.

Group.	Treatment.	Number of rabbits.	CONCENTRATION OF FATTY ACIDS IN LIVER.				IODINE NUMBER OF FATTY ACIDS.			
			Mean and S. D.	Groups compared.	*t	†p	Mean and S. D.	Groups compared.	*t	†p
1	No treatment	8	3.138 ± 0.305	1,2	4.011	<0.01	117.62 ± 8.52	1,2	5.85	<0.01
2	Anterior pituitary extract.	7	6.302 ± 2.24	2,3	0.305	0.65	85.31 ± 12.7	2,3	0.45	0.65
3	Choline feeding and anterior pituitary extract.	6	6.02 ± 0.352	1,3	17.08	<0.01	81.53 ± 16.98	1,3	5.467	<0.01

*t = Test of significance (Fisher) = $\frac{x_1 - x_2}{\sqrt{\frac{\sum d_i^2 + \sum d_j^2}{n_1 + n_2}}}$ $\sqrt{\frac{(n_1+1)(n_2+1)}{n_1+n_2+2}}$ where x_1, x_2 = mean of two groups.
†p = Probability that as great differences in concentrations of fatty acids and iodine numbers would be observed if animals were chosen at random.
 $\sum d_i^2, \sum d_j^2$ = sum of standard deviations.
 n_1, n_2 = number of observations minus 1.

DISCUSSION.

From the experiments which have been reported, two facts emerge which are worthy of consideration. That certain fractions of the anterior pituitary are effective in bringing about an increase in the fatty acid concentration of the liver is evident. This fatty change is, however, not as marked as has been reported by Best and Campbell (*loc. cit.*) in rats where they obtained, in extreme cases, as much as a 600 per cent increase. The species of animal and the conditions of our experiments were not identical and hence strict comparisons are not possible. Further, this fatty infiltration produced by the anterior pituitary extracts is not prevented by choline feeding. Best and his co-workers have previously shown that choline feeding is capable of preventing the fatty infiltration of liver produced by dietetic means. These observations raise interesting issues and enable us to speculate on the probable mechanism of fatty infiltration brought about by anterior pituitary extracts on the one hand, and fat and cholesterol feeding on the other. Fat deposition in the liver is due either to the fact (1) that excess of fat is being presented to it, (2) that the rate of oxidation or the retention of fat by the liver-cells is changed, or (3) that the factors which are normally responsible for the transportation of fat from the liver are not operating with sufficient speed. It is interesting to investigate which of these processes are affected by anterior pituitary injection, fat feeding, and choline administration. Our knowledge with regard to several aspects of fat metabolism is still incomplete and unless these are cleared up, it will be futile to attempt a satisfactory explanation. From the extensive studies carried out by the Toronto group in this field, certain tentative deductions may be drawn. Evidence is available in the published literature that choline effect is not apparently due to an action on the absorption or excretion of fat but probably due to a stimulation of the oxidative mechanism of the liver-cells. The effect of choline is chiefly exerted on the neutral fats and choline esters in the liver only, and the fat depôts in other parts of the body appear to be unaffected by it. On the other hand, it has been shown by Schäfer (1931), and Best and Campbell (*loc. cit.*) that the fatty infiltration caused by anterior pituitary extract is probably due to a mobilization of liver-fat from the depôts. In our experiments, the significant lowering of the iodine numbers after anterior pituitary provides another evidence in support of this statement. It is, therefore, logical to conclude that the fatty infiltration in the case of anterior pituitary is not produced through the same mechanism as operates in the case of fat feeding. During recent years, much work has been done in establishing a relationship between the anterior pituitary and the pancreas (Houssay, 1936; Barnes and Regan, 1933; Anselmino, 1935). It is possible that the accumulation of fat after anterior pituitary is only an expression of an imbalance between these two glands leading to a defective carbohydrate metabolism. This, in its turn, is bound to affect fat metabolism in the liver which is recognized to be the most important site for the de-saturation of fatty acids and the formation of ketone bodies.

CONCLUSIONS.

1. A fatty infiltration of the liver may be produced within 24 hours in rabbits by the injection of a single large dose of anterior pituitary extract. The average

A 0.05 per cent saline solution of the saponin isolated from *Baringtonia acutangula* gave the results given in Table II:—

TABLE II.

Serial number.	c.c. R. B. C.	c.c. saponin, 0.05 per cent.	c.c. N-saline.	UNFILTERED.			FILTERED.			REMARKS.
				1 hour.	2 hours.	21 hours.	1 hour.	2 hours.	21 hours.	
1	1	1.00	—	+++++	+++++	+++++	—	—	—	Almost immediate hæmolytic in the first three tubes.
2	1	0.50	0.50	++++	++++	++++	—	—	—	
3	1	0.25	0.75	+++	++++	++++	—	—	—	
4	1	0.10	0.90	+	++	+++	—	—	—	
5	1	0.05	0.95	—	—	++	—	—	—	

A very dilute solution of cyclamine in normal saline (0.001 per cent) gave almost similar results (Table III):—

TABLE III.

Serial number.	c.c. R. B. C.	c.c. saponin, 0.001 per cent.	c.c. N-saline.	UNFILTERED.			FILTERED.			REMARKS.
				1 hour.	2 hours.	21 hours.	1 hour.	2 hours.	21 hours.	
1	1	1.00	—	+++++	+++++	+++++	—	—	—	No. 1 starts almost immediate hæmolytic.
2	1	0.5	0.5	++++	++++	++++	—	—	—	
3	1	0.2	0.8	—	—	+	—	—	—	
4	1	0.1	0.9	—	—	—	—	—	—	
5	1	0.05	0.95	—	—	—	—	—	—	

It will be seen from the above that the Seitz filtrates of the saponin solutions are rendered absolutely non-hæmolytic, the unfiltered solutions showing the usual

hæmolytic activity. It should be noted in this connection that the Seitz filter can effectively remove the hæmolysin only in dilute solutions and this dilution in turn depends upon the nature of the substance. For instance, though the hæmolysin is completely removed from a 0·01 per cent solution of cobra venom, when a 0·1 per cent solution was used, the filtrate proved to be almost equally hæmolytic as unfiltered solution. A 0·01 per cent solution of cyclamine filtered and unfiltered was found to be strongly and almost equally hæmolytic. The same also holds good of a 0·5 per cent Merck's saponin solution.

Bacterial hæmolysins.—Under this head hæmolysins of cholera vibrio and of streptococcus hæmolyticus were studied. Cholera vibrios were grown on 1 per cent peptone solution and the streptococcus on serum broth.

A twenty-four hours' old culture was used in each case. A 3 per cent suspension of washed sheep's R. B. C. was employed in studying the hæmolysis. In each of the cases a culture from a non-hæmolytic strain of the respective organisms was also used as control (*see* Tables IV-A and IV-B):—

TABLE IV-A.

Serial number.	c.c. R. B. C.	c.c. culture fluid.	c.c. N-saline.	HÆMOLYTIC (UNFILTERED).			HÆMOLYTIC (FILTERED).			NON-HÆMOLYTIC.		
				$\frac{1}{2}$ hour.	2 hours.	21 hours.	$\frac{1}{2}$ hour.	2 hours.	21 hours.	$\frac{1}{2}$ hour.	2 hours.	21 hours.
Cholera vibrio.	1	0·3	0·5	0·2	+++++	+++++	+++++	—	—	—	—	—
	2	0·3	0·2	0·5	+++++	+++++	+++++	—	—	—	—	—
	3	0·3	0·1	0·6	+++++	+++++	+++++	—	—	—	—	—
	4	0·3	0·05	0·65	++	++++	+++++	—	—	—	—	—
	5	0·3	..	0·7	—	—	—	—	—	—	—	—

TABLE IV-B.

Streptococcus hæmolyticus	1	0·3	0·5	0·2	—	++++	+++++	—	—	—	—	—
	2	0·3	0·2	0·5	—	++++	+++++	—	—	—	—	—
	3	0·3	0·1	0·6	—	++	++++	—	—	—	—	—
	4	0·3	0·05	0·65	—	+	+++	—	—	—	—	—
	5	0·3	..	0·7	—	—	—	—	—	—	—	—

It is clear from the above that cholera and streptococcal hæmolysins are effectively removed by Seitz filter and we believe that other bacterial hæmolysins will behave in a similar manner.

COMPONENTS OF A HÆMOLYTIC SYSTEM.

In order to see what effect this Seitz filtration has upon the components of a simple hæmolytic system, guinea-pig's diluted complement 1 to 30 containing 5 M. H. D. and diluted anti-sheep amboceptor 1 in 6,000 were passed through Seitz filter and the effect noted. The results are given in Table V:—

TABLE V.

	Serial number.	c.c. R. B. C.	c.c. N-saline.	c.c. complement.	Dilution of complement.	15 minutes.	$\frac{1}{2}$ hour.	1 hour.	21 hours.
Sheep's R. B. C. sensitized and guinea-pig's complement unfiltered.	1	0.3	0.6	0.1	1/30	+++++	+++++	+++++	+++++
	2	0.3	0.6	0.1	1/40	+++	++++	++++	++++
	3	0.3	0.6	0.1	1/50	—	+	+	++
	4	0.3	0.6	0.1	1/60	—	—	—	—
Sheep's R. B. C. sensitized complement filtered.	5	0.3	0.6	0.1	1/30	—	—	—	..
	6	0.3	0.6	0.1	1/40	—	—	—	—
	7	0.3	0.6	0.1	1/50	—	—	—	—
	8	0.3	0.6	0.1	1/60	—	—	—	—
Sheep's R. B. C. incubated with filtered amboceptor, complement unfiltered.	9	0.3	0.6	0.1	1/30	—	—	—	—
	10	0.3	0.6	0.1	1/40	—	—	—	—
	11	0.3	0.6	0.1	1/50	—	—	—	—
	12	0.3	0.6	0.1	1/60	—	—	—	—

The above results indicate that both complement and amboceptor in dilute solutions are effectively removed by this filter.

The effect of Seitz filtration upon serum hæmolysin was also observed in a few cases.

A 3 per cent suspension of human R. B. C. in N-saline was prepared and the effect of fresh sheep's serum filtered and unfiltered upon it was noted (*see* Table VI) :—

TABLE VI.

Serial number.	c.c. R. B. C.	c.c. serum.	c.c. N-saline.	UNFILTERED SERUM.			FILTERED SERUM.		
				HÆMOLYSIS.			HÆMOLYSIS.		
				$\frac{1}{2}$ hour.	2 hours.	21 hours.	$\frac{1}{2}$ hour.	2 hours.	21 hours.
1	0.3	0.5	0.2	+++++	+++++	+++++	+	+++	+++
2	0.3	0.2	0.5	+++	+++	+++++	—	—	+++
3	0.3	0.1	0.6	+	++	+++++	—	—	—
4	0.3	0.05	0.65	—	—	±	—	—	—

It appears that undiluted serum though it has lost much of its activity by filtration still retains it to a slight extent. This experiment was, therefore, repeated with the serum diluted 1 : 1 with normal saline. The results are given in Table VII :—

TABLE VII.

Serial number.	c.c. R. B. C.	c.c. serum dilution 1 : 1.	c.c. N-saline.	UNFILTERED SERUM.			FILTERED SERUM.		
				HÆMOLYSIS.			HÆMOLYSIS.		
				$\frac{1}{2}$ hour.	1 hour.	2 hours.	$\frac{1}{2}$ hour.	1 hour.	2 hours.
1	0.3	0.5	0.2	+++	+++	+++	—	±	+
2	0.3	0.2	0.5	±	+	++	—	—	—
3	0.3	0.1	0.6	—	—	±	—	—	—
4	0.3	0.05	0.65	—	—	—	—	—	—

Note.—

+++++ = complete hæmolysis. ± = doubtful hæmolysis. — = no hæmolysis.

The hæmolysin, therefore, is more or less completely removed from the diluted serum by this process of filtration. This is quite in keeping with what was observed in case of the members of the saponin group.

DISCUSSION OF THE RESULTS.

The Seitz filter consists of a disc of asbestos and it appears that it acts not merely by holding back the micro-organisms mechanically but has a distinct surface action on a variety of substances. Coplan (1913-14) produced evidence to show that asbestos fibre possesses a number of activities which stamp it as anything but an indifferent and inert substance. He showed that toxins including those derived from *C. diphtheria*, *B. tetani*, *B. tuberculosis*, and *B. mallei*, certain antitoxic sera, specific agglutinins, certain proteins, pigments, and colloids including starch solutions, diastase, cobra venom solution, and the complement and amboceptor of a hæmolytic system, appeared to readily disappear from the solution when kept in contact with asbestos. It is evident from the above that asbestos fibre of which Seitz filter is composed has a distinct surface action on a variety of substances and it acts either by eliminating the active principle by the process of adsorption or by altering its nature altogether. Which of the processes is active in a particular case, it is difficult to say just at present. When the concentration of the active substance is low it may be removed altogether in the majority of cases but with more concentrated solutions it has hardly any noticeable effect, probably because the thin layer of asbestos can cope with only a limited amount of the active substance when it is filtered through it under suction, i.e., when the contact is only for a short time. Our experiments have shown that bacterial hæmolytins are totally eliminated or in some way rendered inert by this process. When, therefore, one is working with bacterial exotoxin or hæmolytins which are present in very small amounts or diluted solutions of other substances the use of Seitz filter must necessarily be avoided. Experiments were also done with a view to see what effect, if any, filtration through Berkefeld filters or through a layer of kieselguhr has upon the hæmolytins studied here. It was found that while kieselguhr has no appreciable action on the hæmolytins, Berkefeld candles have only a slight action on these.

SUMMARY AND CONCLUSIONS.

1. Dilute solutions of saponin (Merck's), cyclamine, and the saponin isolated from *Baringtonia acutangula* lose their hæmolytic property when passed through a Seitz filter.
2. Bacterial hæmolytins are similarly affected.
3. Diluted complement and amboceptor are inactivated by this process of filtration.
4. Concentrated solutions of the active substances are comparatively unaffected.
5. The use of the Seitz filter in investigation relating to bacterial toxins and when dealing with dilute solutions is attended with serious difficulties.

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USE OF NON-SPECIFIC SUBSTANCES IN THE PRODUCTION OF TETANUS AND DIPHTHERIA ANTITOXIN.

BY

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(From the Serum Department, Bengal Immunity Research Laboratory, Calcutta.)

[Received for publication, March 17, 1938.]

THE use of the non-specific substances in enhancing the activity of the antigens was first observed by Ramon (1925). He was led to the track by the finding that horses having an abscess at the site of the last injection due to infection with skin organisms yielded a very high-titre anti-diphtheria serum in comparison to the others having no such abscess. Experimenting with a number of non-specific substances after this finding, with a view to elucidate the nature of this action, he found 'tapioca' to be the best adjuvant and concluded that the effect of those substances was to promote slow absorption and elimination of the antigens, which could thus exert their fullest antigenic activity.

After these experiments Ramon and Descomby (1925) reported the production of high-titre diphtheria and tetanus antitoxin by using 1 per cent tapioca powder with the respective antigens. Glenny *et al.* (1926) reported very good results in the production of diphtheria antitoxin with alum precipitated toxoids. Following those works, Ramon *et al.* (1937) and Glenny *et al.* (1931) published a number of papers on the subject, dealing with its various aspects. With the exception of tapioca and alum, other substances were also reported to give good results. Schmidt (1931) and others obtained better antitoxin production with diphtheria toxoid in combination with aluminium hydroxide than with the toxoid alone. Ramon and his collaborators used calcium chloride with good results too. This principle has also found application in the use of the so-called 'vaccins associées', where a number of antigens are used together—one acting as the adjuvant of the other.

Recently, Ramon, Lemetayer and Richou (1937) tested the efficacy of a number of non-specific substances in increasing the antitoxin production in different types of animals. In guinea-pigs and rabbits they found that the incorporation of lanolin and olive oil or vaselin with the respective antigens produced the corresponding antitoxin several times as much as that produced by the antigens alone. The same result was obtained with saponin which caused a local inflammation. In sheep, tetanus anatoxin with emulsified vaselin and sterol or with lanolin produced an antitoxin about 1,000 times as much as that produced by anatoxin alone. In horses, anatoxin mixed with vaselin and cholesterol or vaselin and lanolin produced

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of toxoid mixed with powdered tapioca, followed by another series of injections of heavy doses of toxoid to which enough calcium gluconate was added to make its concentration 1 per cent. The results are recorded in Tables I and II. The titre is expressed in International Units. The maximum dose of toxoid injected to the horses varied from 500 to 550 c.c.

TABLE I.

Titration of horses given five injections of toxoid and tapioca and then another five injections of toxoid and calcium gluconate.

Horse number.	Titre at the commencement.	Titre after the fifth injection.	Titre after the tenth injection.
492	50	100	400
478	300	300	750
286	200	500	2,500
540	300	500	4,000
539	100	750	2,200
537	100	500	1,200
515	200	600	1,500
484	100	750	1,000
461	100	300	400
504	400	750	2,800
510	300	600	2,000
499	50	500	750
AVERAGE ..			1,625

TABLE II.

Titre of the horses treated with antigen only.

Horse number.	Titre at the commencement.	Titre after the fifth injection.	Titre after the tenth injection.
522	100	400	800
523	200	500	700
538	100	200	400
241	200	300	500
533	300	700	1,000
463	200	400	300
AVERAGE ..			617

DISCUSSION.

The data recorded in Tables I and II show that the horses which received injections of toxoid mixed at first with tapioca and then with calcium gluconate responded much better than horses which received toxoid only. The average titre of the first group of horses was about 1,625 I. U., whereas the average titre of the second group of horses which received the tetanus toxoid alone was 617 I. U. These results are in agreement with the conclusion stated in our previous paper. In the immunization of horses for the production of diphtheria antitoxin Ramon and Lemetayer (1932) recorded that by injecting horses with toxoid mixed first with powdered tapioca (1 per cent) and then with calcium chloride (1 per cent) not only the titre of the serum of the individual horses could be raised to a high value but it could also be maintained at that value for a fairly long time.

In our experiments, however, the high titre attained in the horses belonging to group I could not be maintained for long. It began to fall rapidly after the first two or three bleedings.

Further work is in progress on the maintenance of titre in horses under immunization for the production of tetanus antitoxin.

ACKNOWLEDGMENTS.

We offer our grateful thanks to Dr. B. N. Ghosh, D.Sc., for his advice and guidance and to the Managing Director, Bengal Immunity, for the facilities offered to carry out this work. We are also indebted to Mr. J. M. Datta for assisting us in this work.

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APPLICATION OF ANALYSIS OF VARIANCE TO MORTALITY RATES.*

BY

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EPIDEMIC diseases exhibit a variability in incidence during the different parts of a year and also from year to year. The criteria that may, therefore, be taken into account in describing the course of an epidemic disease are :—

- (1) the intensity of incidence,
- (2) the distribution of its prevalence within the year, and
- (3) its variation from year to year.

For measuring intensity the mean or median incidence may be taken for the period under investigation taking weekly, monthly, or yearly rates for this purpose. The mean weekly or monthly values, when plotted out on graph paper, give a picture of the mean seasonal variation. Similarly, yearly variation may be judged by calculating yearly mean values and plotting them out against time as the other variable.

In comparing one place (or disease) with another what is usually done is to compare these characteristics separately. The disadvantages in this procedure are that :—

1. The method of comparison is purely by inspection and is, therefore, liable to lead to widely varying results at the hands of different investigators.

* Read at the *Statistical Conference*, Calcutta, 1938.

2. The question of random variation of the three characteristics is not taken into account.

3. Each characteristic can only be judged independently of the other two. The question of the simultaneous variation of two factors, e.g., change of the seasonal curve from year to year, cannot be allowed for.

4. There are no objective tests for establishing similarity or otherwise between two diseases or places which are being compared.

5. If the number of places to be compared is large then comparison by mere inspection becomes wellnigh impossible.

For the purpose of comparison, therefore, we want a method by which we can numerically assess the various characteristics independently of one another, estimate the errors of these measures due to random variation and provide tests by which to compare places (or diseases) after taking all the three factors into consideration.

Problems of a similar nature in other fields of research have been solved by the application of the method of analysis of variance, which has been devised by Prof. R. A. Fisher. The application of the method over the data relating to one place consists essentially in splitting up the total variability of incidence round the mean into the following parts:--

- (1) Seasonal variability or between months.
- (2) Cyclical variability or between years.
- (3) Residual variability.

The first and second constitute quantitative expressions of the effect of factors which come into operation at special seasons of the year or during particular years respectively. A high seasonal variability may point to factors, such as climatic variation or the occurrence of festivals or other causes producing large scale movements of population, agricultural labour for instance, as being responsible for the recurrence of disease at particular seasons year after year. A large yearly variance may, similarly, suggest that the area concerned is of the epidemic or endemic-epidemic type, so that years of high incidence are followed by periods of comparative quiescence.

The third portion of variability is the result of all other causes producing fluctuation in cholera mortality in the centre. Fisher's 'Z-test' is designed to ascertain whether the mean variances due to months and years are significant, that is, whether in regard to the particular place, seasonal and cyclical factors are important. The residual, if it is large, suggests that the seasonal and yearly factors are not sufficient to explain the fluctuation in the disease incidence and that, therefore, further investigation is required. A low residual would, on the other hand, suggest that the monthly and yearly factors are the important ones and that attention must, therefore, be directed towards the elucidation of the causes associated with these factors.

In Table I the cholera mortality rates per 10,000 population for Amtoli thana in the district of Backergunj, Bengal, are analysed by the method of analysis of variance (the *Appendix* sets out the contingency table).

TABLE I.

Analysis of variance table for Amtoli thana, Backergunj district, Bengal.

Source of variation.	Degrees of freedom.	Sum of squares.	Mean variance.
Months ..	11	4,988.5779	453.5071
Years ..	31	2,319.1481	74.8112
Residual ..	341	11,718.0235	34.3637

Both seasonal and yearly effects are significantly different from the residual when judged by the Z-test.

Analyses similar to the above were carried out for a number of centres and the seasonal, yearly, and residual variations were separated.

The purpose of this study was to obtain homogeneous areas of fairly large size for epidemiological investigations and the next step, after analysing the variance of individual thanas, was to combine those which were contiguous and which exhibited similarity. For this purpose a number of thanas, which appeared to be similar on inspection of the results of bivariate analyses, were combined and a trivariate analysis was carried out, the total variability being split up into the following :—

Source of variation.

1. Between means of thanas. 2. Between means of months. 3. Between means of years.

Interactions.

4. Between thana and month. 5. Between thana and year. 6. Between month and year. 7. Residual.

The mean variances represented by (1), (4), and (5) should not be significant when tested against the residual (7), if the area is to be considered homogeneous. If variance (1) is significant, it indicates that the mean incidence of the disease for the whole period under consideration differs from place to place to such an extent that we cannot attribute these differences solely to random causes. Absence of significance for the interactions (4) and (5) indicates that there is no significant difference between place and place as regards seasonal or yearly fluctuations, which is what we want. Lastly, the mean variances (2), (3), and (6) represent the variations of mean monthly or yearly incidences for the area as a whole and they, therefore, form characteristics of the area in question. From the point of view of similarity of the component elements of a homogeneous area, it is immaterial whether they are significant or not.

The results of trivariate analysis of the cholera mortality rates per 10,000 population of a group of three centres in Backergunj district (Golachippa, Patwakhali, and Amtoli) are shown in Table II :—

TABLE II.
Results of the trivariate analysis of variance.

		Degrees of freedom.	Sum of squares.	Mean squares.
<i>Source of variation.</i>				
1. Means of months	..	11	15,869·3582	1,442·6689
2. Means of years	..	31	6,047·9694	195·0958
3. Means of places	..	2	138·8506	69·4253
<i>Interactions.</i>				
4. Year and months	..	341	23,435·2844	68·7252
5. Months and place	..	22	863·8074	39·2640
6. Place and year	..	62	1,017·7970	16·4161
7. Residual	..	682	6,836·2136	10·0238
TOTALS	..	1,151	54,209·2806	..

The mean squares are all significant by the Z-test. Hence these three centres cannot be considered to be homogeneous with respect to cholera mortality. Similar analyses were carried on several groups of centres and it was found that the tests were too rigorous to admit of even three contiguous centres being combined.

At this stage the question of application of Z-test to these data was investigated. There are certain theoretical considerations involved in the application of the methods outlined above. The assumption is that each mortality rate (see *Appendix* for contingency table) is a random sample from a separate normally distributed infinite population of values and that, while these populations may differ from one another with respect to their means, their variances round the respective means have the same value. The first question that presents itself is whether the assumption of normality can be supposed to hold good in our case. It has been shown that a certain amount of departure from normality is permissible with respect to the application of Z-test. However, in our case the probability of death (say p) is generally so small that even when the population (say n) is large—our figures range from about 20,000 to 300,000—or tends to infinity, binomial $(p+q)^n$ approximates more to a Poisson's than to a normal distribution. Under the circumstances, we should expect that if replicate values were available for our

mortality rates the variation among them would be of the Poisson type. We have no real replicates. Weekly rates for the same month, year, and place are the best data we can think of.

If the cell populations sampled were normally distributed the means and standard deviations of successive samples should not show any significant correlation. Weekly figures for certain places were available from 1930 onwards. The correlations between mean and standard deviation were calculated for a number of centres and they were in all cases significant.

If the assumption is made that the distributions of the populations sampled are likely to approximate to Poisson's, then transformation of the data by taking their square roots should suffice to meet the case. This was tried but the correlation co-efficients remained above the significance level. Cube-root and even tenth-root transformations failed to bring the correlations below significance level. Trigonometric transformation, however, brought it down practically to zero but the transformed data gave a wrong interpretation to the original figures because after transformation high values of the latter gave zero or even negative values and vice versa.

These considerations suggest the inapplicability of the Z-test and raise the question of a modification of this method, which could be applicable to these types of data.

For our purpose the procedure followed was to work out the contributions to total variance of each of the sources of variation. In the group already mentioned the contributions were as given in Table III :—

TABLE III.

Percentage contribution for each source of variation.

			Percentage contribution.	
(1) Month	(1,442·6689—10·0238)/96 = 14·9234	29·115
(2) Year	(195·0958—10·0238)/36 = 5·1408	10·030
(3) Place	(69·4253—10·0238)/384 = 0·1547	0·302
(4) Month and year	(68·7252—10·0238)/3 = 19·5671	38·175
(5) Month and place	(39·2640—10·0238)/32 = 0·9138	1·783
(6) Year and place	(16·4161—10·0238)/12 = 0·5327	1·039
(7) Residual	10·0238	19·556
			TOTAL ..	51·2563

The last column shows the percentage contribution of each source. If the contribution from any one of the sources (3), (5), and (6), was more than 5 per cent of the total variance then the places were considered significantly different from each other. Although the method lacks theoretical justification it provides us with an objective test for establishing heterogeneity in epidemiological investigations. We used this method for combining places in a number of cases. Although by the Z-test the combined areas always showed heterogeneity amongst their component elements, means and standard deviations of month, years, and residuals did not exhibit any appreciable divergence from place to place in the homogeneous groups evolved by the method described above, as may be seen from the figures given in Table IV which relate to the group analysed in Table III :—

TABLE IV.

Place.	Mean mortality per 10,000.	Seasonal standard deviation.	Yearly standard deviation.	Residual standard deviation.
Amtoli	2.44	3.62	1.84	5.86
Patwakhali ..	3.27	3.61	2.16	5.59
Golachippa ..	3.02	4.32	1.88	4.84

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We had to refer to Dr. J. O. Irwin of the London School of Tropical Medicine for guidance in connection with the theoretical considerations involved in these studies. We are grateful to him for the great interest he took in this work and for giving us the benefit of his advice.

APPENDIX.

The contingency sheet of monthly cholera mortality rates per 10,000 for Antoli thana, Backergunj district.
Period 1901-1932.

Year.	January.	February.	March.	April.	May.	June.	July.	August.	September.	October.	November.	December.	Totals.
1932	129	86	1,308	527	316	86	13	19	5	0	0	0	2,669
1931	384	83	296	160	53	19	29	0	0	0	19	169	1,203
1930	148	10	74	315	44	15	20	0	25	26	0	84	785
1929	40	25	115	30	20	55	0	0	0	0	0	521	806
1928	143	122	835	865	188	76	15	0	0	10	0	66	2,320
1927	284	171	1,303	337	57	10	16	0	0	0	10	212	2,420
1926	0	5	436	84	37	0	5	0	0	0	0	53	620
1925	32	27	208	139	121	0	0	0	0	0	0	21	418
1924	168	65	538	331	163	11	0	0	0	0	0	27	1,243
1923	464	44	1,774	602	61	11	0	0	11	0	17	155	3,139
1922	6	17	51	410	157	6	0	0	0	0	0	343	990
1921	773	1,305	852	52	46	17	0	0	0	0	0	17	3,062
1920	169	41	58	41	64	54	6	12	0	0	105	268	822
1919	53	2,025	2,966	776	373	89	11	0	0	0	0	18	6,311
1918	12	391	669	602	96	0	12	0	0	0	0	42	1,734
1917	80	18	135	147	25	12	0	6	0	0	0	12	435
1916	12	19	231	287	51	0	0	0	0	0	0	131	730
1915	191	280	6,537	1,296	13	13	57	0	0	0	0	19	8,406
1914	136	362	1,087	899	116	0	0	0	0	0	45	201	2,846
1913	33	1,220	3,231	487	0	13	0	0	0	0	0	79	5,063
1912	262	222	504	343	87	0	0	0	0	0	0	0	1,418
1911	970	370	377	441	130	0	14	0	0	0	0	27	2,199
1910	982	348	599	272	202	7	7	0	0	0	0	703	3,113
1909	21	7	134	742	869	28	7	21	0	0	0	1,074	2,903
1908	122	158	1,541	688	143	0	0	0	0	0	0	72	2,724
1907	960	2,021	9,591	3,025	51	15	7	0	65	0	0	204	15,939
1906	472	664	1,963	768	229	266	89	0	89	0	0	1,173	5,712
1905	524	7	427	127	75	0	0	0	15	0	0	150	1,340
1904	312	304	578	251	0	0	0	0	0	0	190	533	2,83
1903	788	240	1,151	379	51	0	0	0	8	0	0	270	2,890
1902	1,044	612	1,240	366	16	16	0	0	8	0	0	330	5,572
1901	407	120	806	861	207	0	8	0	0	0	0	1,364	3,773
Totals	10,021	11,299	41,705	16,610	3,903	823	309	58	218	38	386	8,329	93,759

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ELECTROPHORESIS AND METABOLISM OF SOME VIBRIO STRAINS IN RELATION TO VARIABILITY AND CHEMICAL CLASSIFICATION.

BY

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BRUCE WHITE (1935) brought about the transformation of vibrio strains from the smooth to the rough state, and sent some of them to this laboratory for examination. His method consisted of the exposure of young smooth cultures to their homologous activated antisera. Most of the organisms were lysed by this treatment, but among the survivors there was a considerable proportion of the rough type, and these were found to be stable on plating. By the further treatment of such 'R' colonies with activated anti-'R' sera, races designated as ρ types were obtained, and these in turn showed greatly reduced agglutinability with the anti-'R' sera. The 'R' strains were serologically more generalized than the 'S' strains, in that serological differences between the latter were found to have disappeared.

It was of interest to examine these strains to determine possible differences between the 'S' and 'R' homologues with respect to chemical structure, electrophoresis, and metabolism. The methods, in each instance, were the same as those previously described (Linton, Mitra and Shrivastava, 1934; Linton, Mitra and Seal, 1936a and b; Linton, Mitra and Mullick, 1936a and b).

In Table I are given the results of a study of chemical structure and metabolism of 26 strains (including the 'R' derivatives in seven cases and the ρ derivative in one case) obtained from Bruce White, together with seven strains which we had obtained from different sources in India. The sources of these strains correspond, as usual,

TABLE I.

Strains.	Source and date of isolation.	Origin.	Received from	Linton's chemical group.	Respiration.	Aerobic glycolysis.	Metabolism group.
Inaba ..	Japan, old strain	Cholera case	Mr. Bruce White	I	12.8	3.2	I
Inaba R	Derived from Inaba.	"	IV	13.1	0.0	IV
Shillong 1077 ..	Shillong, Col. Morison, 1934	Cholera case	"	I	12.6	7.3	I
Shillong 1077 R	Derived from Shillong 1077.	"	IV	10.9	0.5	IV
Shillong 1077 ρ	Derived from Shillong 1077 R.	"	IV	11.1	0.0	IV
Kasauli 11 ..	Cent. Res. Inst., Kasauli, 1932.	Cholera case	"	I	12.2	6.8	I
*Kasauli 11 R	Derived from Kasauli 11.	"	IV	11.1	0.4	IV
Kasauli 92/1 ..	Cent. Res. Inst., Kasauli, Capt. Ahuja, 1934.	Cholera case	"	I	12.6	6.8	I
Kasauli 92/1 R	Derived from Kasauli 92/1.	"	IV	10.5	0.0	IV
Nanking 32/77 ..	Dr. Yang, 1932	Cholera case	"	I	12.4	7.7	I
Nanking 32/77 R..	..	Derived from Nanking 32/77.	"	IV	9.8	0.2	IV
Nanking 32/101 ..	Dr. Yang, 1932	Water	"	IV + V	13.2	3.3	V
Nanking 32/101 R	..	Derived from Nanking 32/101.	"	IV	10.7	0.0	IV
Nanking 32/110 ..	Dr. Yang, 1932	Water	"	IV + V	11.2	2.1	V

Nanking 32/110 R	..	Derived from Nanking 32/110.	"	IV	10.4	0.0	IV
Shillong 610 ..	Shillong, Col. Morison, 1934	Cholera case	Major Pasricha	I	13.2	7.1	I
Hikojima ..	Japan, old strain	"	Col. Taylor	I	12.4	6.8	I
Shillong 653 ..	Shillong, Col. Morison, 1934	"	Col. Anderson	II	6.9	4.4	II
Habiganj 984 ..	Shillong, 1935	"	"	II	7.6	4.8	II
Habiganj 3065 ..	" 1935	"	"	II	7.0	4.5	II
200 ..	Calcutta School of Tropical Medicine, Major Pasricha, 1937.	"	Major Pasricha	II	7.1	5.3	II
334 ..	Do., do., 1937	"	"	II	7.2	5.0	II
Nanking 32/123 ..	Dr. Yang, 1932	"	Col. Taylor	VI	9.1	6.0	VI
Nanking 32/124 ..	" 1932	"	"	VI	9.4	5.5	VI
Kasauli 73 G ..	Cent. Res. Inst., Kasauli	"	"	VI	8.3	4.8	VI
Ogawa ..	Japan, old strain	"	"	VI	9.8	5.0	VI
2252 ..	Calcutta School of Tropical Medicine, Major Pasricha, 1936.	Healthy human carrier.	Major Pasricha	V	8.8	2.2	V
El Tor 34/D/19 ..	Quar. Camp., El Tor, 1934	Healthy pilgrim	Col. Taylor	V	9.1	1.6	V
Doorenbos 34/11	Dr. Doorenbos, 1934	"	Mr. Bruce White	V	9.4	1.4	V
Doorenbos 34/13	" 1934	"	"	V	10.3	1.8	V
Nanking 32/103 ..	Nanking, 1932	Water	Col. Taylor	III	5.2	1.8	III
Nanking 32/105 ..	" 1932	"	"	III	5.7	2.4	III
Nanking 32/109 ..	" 1932	"	"	IV	11.0	0.0	IV

* Mr. Bruce White was not sure of the stability of roughness of this strain.

with their chemical structure. Case strains belong to groups I, II, and VI and water and carrier strains to groups III, IV, and V. The former groups possess protein I and the latter protein II. As in our previous work, the group VI strains are old case strains, the difference between this group and groups I and II lying in the polysaccharide. Freshly isolated case strains, in our experience, always belong to group I or II (Linton, Shrivastava, Seal and Mookerji, 1938).

In their metabolism, the strains correspond exactly to their chemical structure, and indicate again the close connection between these two characteristics of the organism. The figures for the ranges of each group have been given in detail in two previous publications (Linton, Mitra and Mullick, 1936*a* and *b*).

In regard to the original and derived strains of Bruce White, several points of interest are apparent. In the first place, the method which he has used to bring about roughening has at the same time been accompanied by a change in chemical structure. In each case this has led to the derived strain now falling into group IV, and possessing protein II and polysaccharide I. At the same time, as we have found previously, the metabolism of the derivatives changes, and becomes the same as that of all previous group IV strains we have studied. In the case of group IV strains of our present study this change is especially striking, since it involves the practical disappearance of aerobic glycolysis, which drops from an average of 7.3 mm. CO₂ to 0.2 mm. Respiration, as we have previously shown, has the same range in group IV as in group I strains.

Certain of the strains received from Bruce White have also been studied on the basis of their potentials as shown by electrophoresis. The results are given in Table II. In each case, the change brought about by the treatment with serum has led to an increased surface potential in the rough survivors. And it is interesting to note that electrophoretically the organisms which are quite distinct from one another in the 'S'-state are often similar or identical in the 'R'-state. This is perhaps the underlying factor to account for the observation of Bruce White who found 'R'-strains serologically more generalized than the 'S'-strains. In the case of Shillong 1077, the ρ strain showed an even higher surface potential than the rough homologue and was very much higher than the original smooth strain.

TABLE II.

Potential differences in millivolts of vibrios at different concentrations of sodium chloride.

Strains.	Linton's chemical group.	CONCENTRATIONS OF SODIUM CHLORIDE.				
		0.0093N.	0.0187N.	0.037N.	0.075N.	0.15N.
Inaba ..	I	-29.91	-23.70	-17.55	-10.57	-4.81
Inaba R ..	IV	-36.94	-29.81	-23.04	-16.82	-10.01
Shillong 1077 ..	I	-28.86	-22.52	-16.12	-10.4	-3.9

TABLE II—*concl'd.*

Strains.	Linton's chemical group.	CONCENTRATIONS OF SODIUM CHLORIDE.				
		0·0093N.	0·0187N.	0·037N.	0·075N.	0·15N.
Shillong 1077 R ..	IV	-34·50	-28·24	-22·08	-16·95	-10·36
Shillong 1077 ρ ..	IV	-36·92	-30·08	-24·96	-18·59	-12·87
Kasauli 11 ..	I	-22·8	-18·04	-13·22	-7·8	- 3·0
*Kasauli 11 R ..	IV	-26·05	-22·32	-18·24	-14·3	-10·4
Kasauli 92/1 ..	I	-30·68	-25·62	-20·75	-15·6	-10·66
Kasauli 92/1 R ..	IV	-36·40	-30·20	-24·22	-18·2	-12·04
Nanking 32/77 ..	I	-26·00	-21·96	-17·78	-13·52	- 9·1
Nanking 32/77 R	IV	-36·66	-29·41	-23·14	-17·03	-10·66
Nanking 32/101 ..	IV + V	-24·05	-19·91	-15·02	-10·4	- 5·2
Nanking 32/101 R	IV	-36·24	-29·38	-23·82	-18·64	-11·05
Nanking 32/110 ..	IV + V	-23·66	-19·85	-16·00	-11·96	- 8·19
Nanking 32/110 R	IV	-36·40	-30·62	-23·84	-18·24	-12·0

*Mr. Bruce White was not sure of the stability of roughness of this strain.

DISCUSSION.

The above results are similar to those previously obtained by Linton, Mitra and Seal (1936c) and show that the roughening obtained by Bruce White brings about the same results as we obtained by treating the organisms by other methods. This same fact is indicated as well in the metabolism and chemical groupings. The changes whether brought about by treatment with antiserum or by passage through various media are the same. Furthermore, the electrophoretic data give a quantitative measure of the degree of roughness, and strains can be more advantageously compared by this method than by agglutination reaction.

The changes in the potential will account in part for the differences in agglutination observed by Bruce White and also for the more generalized serological behaviour of the 'R'-strains. When to them is added the fact that chemical structure and metabolism are also different it is apparent that the strains have become so distinct that the agglutination reaction would be expected to be changed.

SUMMARY.

The chemical structure and metabolic activity of the vibrio strains received from Bruce White and others are closely correlated and changes in chemical structure are accompanied by changes in metabolism. This confirms our previous findings.

Also, the serological changes brought about by treatment with antiserum or by other processes follow changes in chemical structure, metabolism, and surface potential. A shift in the latter is the cause of serological distinction of the 'R'-derivatives from their 'S'-homologues and partly accounts for the uniformity of serological behaviour of the rough vibrio strains in general.

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STUDIES ON MENINGOCOCCUS BACTERIOPHAGE.*

BY

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INTRODUCTION.

MENINGOCOCCUS bacteriophage was accidentally discovered in this laboratory, along with the growth of meningococcus in the solid medium in the course of cultural examination of the cerebro-spinal fluid from a case of meningitis. The routine method of culture in this laboratory is to take the cerebro-spinal fluid directly into ordinary agar and blood agar, at least 5 c.c., for each medium, and to transfer the growth obtained in either, daily, for a week, to ordinary agar as well as to blood agar, to note if there is any growth in ordinary agar in secondary sub-culture (Sen, 1938). The organism isolated from this case (strain 186) was a non-pigmented Gram-negative diplococcus, negative to all sugars generally used for the identification of the genus *Neisseria*, before as well as after passage and was *non-agglutinable* by standard type serum, supplied by Standards Laboratory, Oxford. This description agrees with that of 6-A group of atypical meningococcus (Sen, 1935, 1936a, 1938). Along with the growth of meningococcus, 'plaques' of 'phage or in the words of Asheshov (1933) 'clearings' were observed in the solid medium. Some 'phage broth was poured on to the medium showing the plaques of 'phage and the growth of meningococcus. The whole growth was emulsified in the 'phage broth and kept in the incubator for 24 hours with the idea that if it were actually 'phage it would grow at the expense of the corresponding organism in this medium. The whole mixture was then filtered through an L₃ candle.

Next day two tubes containing 'phage broth medium were taken and inoculated with strain 186. Into one of these, one c.c. of the above filtrate was added, the other being kept as the control, and both tubes were introduced into the incubator.

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There was no visible lysis in the filtrate-added tube after 24 hours' incubation; it remained as turbid as the control. The filtrate-added culture tube was again candle-filtered and the filtrate tested in the above way. Until the fourth transfer there was no apparent change in the filtrate-added culture tube. But on the fifth transfer, there appeared definite lysis as compared with the control. All the meningococcus strains so far isolated in this laboratory (typical as well as atypical) were then tested in the same manner but the filtrate was found to have no lytic effect against any of them except strain 186. *The conclusion drawn was that the filtrate contained bacteriophage against strain 186 and against no other strain of meningococcus.*

MEDIUM USED FOR PASSAGE OF THE 'PHAGE.

Papain digest broth with 0.3 per cent dextrose and 0.5 per cent sodium chloride was first tried for passage of the 'phage, but the growth of meningococcus was not found to be satisfactory in this medium.

The ordinary nutrient broth (using heart muscle instead of meat maceration) containing peptone 2 per cent, dextrose 0.3 per cent, and sodium chloride 0.5 per cent was found more suitable and the growth of the meningococcus was much more abundant than in the former case. After repeated daily passages through some of the locally isolated typical meningococcus showing growth in this medium and through all of the atypical meningococcus including the red and yellow pigmented ones (Sen, 1936b, 1938), for a period of three months, many of them were found to be lysable by the newly isolated 'phage. Foreign strains of meningococcus received through the courtesy of Dr. A. B. Wadsworth and *Neisseria flavescens* strains received through the courtesy of Dr. Sara Branham (1930) were subsequently added to the list of strains for passage. Meningococcus strains received from Dr. Wadsworth as well as some of the locally isolated typical meningococci (as noted above) did not grow in this medium though *Neisseria flavescens* grew well.

The medium was then enriched by adding 0.5 per cent sterile normal horse serum. All the strains which failed to grow previously showed good growth in this enriched medium.

It was doubtful whether this 'phage would continue to grow in a medium containing serum, as it has frequently been reported by other observers that serum and other tissue fluids have the power of adsorption of bacteriophage. This is one of the reasons why parenteral introduction of bacteriophage, particularly inside the blood vessels, is thought to be undesirable and useless. Two sets were, therefore, maintained separately; one set consisting of foreign and some of the locally isolated typical strains (growing only in enriched medium) in 'phage broth containing serum, and the other set comprising the rest of the locally isolated typical (easy growing) and atypical 6-A meningococcus—which is the common finding in Calcutta epidemic—in 'phage broth only. After repeated passages it was found that the virulence of the 'phage, as determined by its power of lysis, had begun to increase in both sets of cultures, so that the idea of the 'phage being inactivated in the presence of serum was proved to be groundless. Separate sets are still, however, being maintained. In the meantime, the search for a common medium was continued, a medium containing no serum but suitable

for the growth of 'phage, as well as for both typical and atypical strains of meningococcus, the addition of serum resulting in the increase of the protein content of the medium.

It was found that only 12 hours were required for the production of complete lysis when four drops of 'phage were added to 8-c.c. cultures in broth tubes. The first lot of meningococcus bacteriophage (lot No. 1) was prepared in 500-c.c. flasks (one flask for each lysable strain) containing 250 c.c. phage-broth *with serum*, 0.3 per cent glucose, and 0.5 per cent salt, against all the strains which had been found lysable, and the 'phage thus prepared was put up in ampoules. The ampoules were tested for the presence of serum by the heat test as well as by Heller's cold nitric acid ring test, both tests giving positive results. All the 'phage ampoules were kept in the ice-chest at a temperature between 5°C. and 10°C.

After lot No. 1 was prepared, the search for a special medium proved successful and all subsequent lots were prepared with this new medium, which is prepared as follows:—

The following ingredients are added to a heart-muscle macerate and the pH is adjusted to 7.8:—

			Per cent.
Bactopeptone	0.2
Sodium chloride	0.5
Dextrose	0.1
Maltose	0.1
Agar-agar	0.01

The medium is distributed in test-tubes in approximately 4½ c.c. amounts. One small piece of coagulated white of egg is added to each test-tube, and the tubes are autoclaved at 10-lb. pressure for half an hour when the final pH is from 7.4 to 7.6.

MODE OF PASSAGE.

At first the mode of operation was to fill racks with 'phage-broth medium in test-tubes containing approximately equal quantities, two tubes being inoculated with each strain. Immediately after the inoculation, ½ c.c. 'phage was added to one of the two tubes for each strain, the other tube remaining as the control. This method was later considered defective, because 'phage does not multiply well unless it is in contact with young growing cultures. The mode of operation was, therefore, changed slightly. Instead of the 'phage being added to the tubes immediately after inoculation, the rack of broth tubes after inoculation was kept in the incubator for three hours and 'phage was then added to alternate tubes containing young cultures.

After daily passage for a few months, the 'phage was found to increase in virulence, complete lysis being then obtained within eight hours by the addition of only one drop of 'phage.

APPARENT DISAPPEARANCE AND REAPPEARANCE OF 'PHAGE.

Suddenly after two months of regular passing the 'phage was found to be missing, that is to say, the filtrate was not able to lyse any of the hitherto lysable

strains. Fortunately the ampoules of lot No. 1 prepared two months before and kept in the ice-chest were still available for testing. One of the ampoules (from lot No. 1) was tested for the presence of 'phage. There was, however, no detectable lysis after 12 hours. It was argued that the organisms might have been lysed very quickly and that the completely clarified tubes (after 'phage action) might have again become turbid owing to the appearance of secondary growth. Hence the racks were examined every hour after the addition of 'phage, to determine whether complete lysis could be seen at any time, and, if so, if it were followed by the appearance of secondary growth. As no incubator was available under the circumstances the racks had to be kept at room temperature (25°C. to 30°C.). The result was complete lysis within four hours, no secondary growth being noticed at any time. This experiment was repeated by Dr. Guha and Mr. Acharya, of this laboratory, and by the author independently, when advantage was taken of the incubator. The result was again found to be unsatisfactory, that is, there was no lysis in any case. Then it was thought that the room temperature was perhaps more suitable than the incubator temperature for the multiplication of the 'phage in this particular case (meningophage). The experiment was then repeated in the laboratory but at room temperature, and this time, with complete success.

It appears, therefore, that the meningococcus bacteriophage acts on meningococcus better at room temperature, i.e., between 25°C. and 30°C., than in the incubator at 37°C.

EFFECT OF TEMPERATURE ON MENINGOPHAGE.

The next experiment was then undertaken to study the effect of temperatures lower than room temperature. Two only of the lysable strains were taken up. All the inoculated tubes were incubated for four hours to ensure uniform growth of the young culture. The results are shown in Table I:—

TABLE I.

Strain number.	Result read after	Ice-chest, 5°C. to 10°C.	Anti-chamber, 15°C. to 20°C.	Room temperature, 25°C. to 30°C.	Incubator, 37°C.	REMARKS.
P 154	24 hours	+++	+++	+++	---	Control tubes showing vigorous growth.
	48 "	+++	+++	+++	---	
P 200	24 "	+++	+++	+++	±--	..
	48 "	+++	+++	+++	---	..

The emulsion and 'phage were kept together in the temperatures noted above.

Note.—The following nomenclature is followed in all the tables:—

- full and vigorous growth of organism.
- ±-- slight difference from the control tube, showing little, if any, lysis.
- +-- definite difference from the control tube, showing the onset of lysis.
- ++- definite lysis but a slight amount of haziness still present.
- +++ complete lysis, showing clear sparkling fluid.

The result is practically the same in both cases.

It appears, therefore, that the development of meningococcus bacteriophage is very much inhibited at incubation temperature but it remains alive and grows in temperatures lower than 37°C. Whether the 'phage dies at that temperature or at a higher temperature remains now to be seen.

THERMAL DEATH POINT OF MENINGOCOCCUS BACTERIOPHAGE.

The 'phage ampoules of lot No. 1 kept about six months in the ice-chest were then subjected to various temperatures—half-an-hour's exposure in each case. The subsequent tests for 'phage action gave the results shown in Table II:—

TABLE II.

Temperature, °C.	Strain P 154.	Strain P 200.	Strain 16.	Strain 508.	Strain NF 155.	REMARKS.
5 to 10 (ice-chest)	}+++	++-	+++	+++	++-	..
15 to 20 (anti-chamber)	}+++	++-	+++	+++	+++	..
25 to 30 (room temperature)	}+++	++-	+++	+++	+++	..
37 (incubator)	}+++	++-	+++	++-	+++	..
45	.. +++	---	+++	++-	++-	..
50	.. +++	---	+++	---	---	} Result seems to vary with different strains.
55	.. +++	---	+-	---	---	
60	.. ---	---	---	---	---	..
75	.. ---	---	---	---	---	..
100	.. ---	---	---	---	---	..
120	.. ---	---	---	---	---	..

See notes under Table I.

It appears that meningococcus bacteriophage is destroyed at and above 60°C. and is inactivated in the case of some strains at temperatures between 45°C. and 55°C. The best action seems to be obtained by keeping it at a temperature of 37°C. or lower.

'Phage was then kept at incubator temperature (37°C.) for 48 hours and its action then tested against the same two strains P 154 and P 200, the mixtures and controls being kept respectively at temperatures of 5°C. to 10°C. (ice-chest), 15°C. to 20°C. (anti-chamber of the ice-chest), 25°C. to 30°C. (room temperature), and 37°C. (incubator) (see Table III).

TABLE III.

Strains.	Result read after	CONTROL AS WELL AS 'PHAGE ADDED TUBES ARE KEPT IN—				REMARKS.
		Ice-chest, 5°C. to 10°C.	Anti-chamber, 15°C. to 20°C.	Room temperature, 25°C. to 30°C.	Incubator, 37°C.	
P 154	24 hours	+++	+++	+++	---	Control tubes in each case had shown vigorous growth after 24 and 48 hours.
	48 "	+++	+++	+++	---*	
P 200	24 "	+++	+++	+++	+--	
	48 "	+++	+++	+++	---*	

See notes under Table I.

* Read below.

Whether the 'phage remained *alive* in contact with the corresponding organism in the incubator for 48 hours, was next to be determined. The 'phage added culture tubes kept at incubator temperature for 48 hours which showed no lysis (i.e., the tubes marked with * in Table III) were then taken out and filtered and the same experiment was repeated with this filtrate, with identical results as those in Table III proving that the meningococcus bacteriophage, though it becomes inactivated at incubator temperature in contact with the corresponding organism, actually remains alive even after 48 hours and can be reactivated at lower temperatures.

The failure of the meningococcus 'phage to produce detectable lysis at incubator temperature appears to be due to the fact that *the rate of growth of the meningococcus 'phage is very much slower than that of the corresponding organism at this temperature and as such its action is not visible, though the 'phage remains alive and active.*

LYSABILITY OF THE DIFFERENT STRAINS BY MENINGOCOCCUS 'PHAGE IN DIFFERENT DILUTIONS.

The power of lysis by the meningococcus 'phage in different dilutions, against all the lysable strains, was next tested in the usual way.

Each strain was inoculated into 12 tubes of 'phage broth medium in approximately equal quantities and kept in the incubator for six hours. Two drops from each dilution (10^{-1} to 10^{-1}) of 'phage were then added to 10 of these inoculated tubes in series, two drops from the original 'phage was added to the 11th inoculated tube and one inoculated tube was kept as control (strain control). All these eleven inoculated tubes after addition of 'phage as well as the control were kept in the room temperature and the result of 'phage action noted after 18 hours (see Table IV).

It appears that the lysability of the strain increases with the time of passage, but there are some amongst the lysable strains against which 'phage grows more quickly. The 'phage originally active only against an atypical strain had become active on strains of recognized serological types.

TABLE IV.

Strains.	Undiluted 'phage.	'PHAGE DILUTIONS.										Control.
		10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸	10 ⁻⁹	10 ⁻¹⁰	
16 ..	+++	+++	+++	++	++	++	++	++	+	---	---	Growth.
154-B ..	+++	+++	++	---	---	---	---	---	---	---	---	"
154-N ..	+++	+++	---	---	---	---	---	---	---	---	---	"
*373 ..	+++	+++	---	---	---	---	---	---	---	---	---	"
200-B ..	+++	+++	++	+	---	---	---	---	---	---	---	"
*419 ..	+++	+++	+++	++	+	+	+	---	---	---	---	"
692 ..	+++	+++	+++	+++	++	++	++	---	---	---	---	"
165 ..	+++	+++	+++	+++	++	++	++	++	+	+	---	"
328 ..	+++	++	---	---	---	---	---	---	---	---	---	"
P 328 ..	+++	+++	+++	+++	---	---	---	---	---	---	---	"
*331 ..	+++	+++	+++	+++	++	++	++	++	---	---	---	"
NF 155 ..	+++	+++	++	+	---	---	---	---	---	---	---	"
NF 157 ..	+++	+++	---	---	---	---	---	---	---	---	---	"

See notes under Table I.

* These are serologically typical locally isolated strains of meningococcus.

5. Out of thirteen lysable strains studied, two drops meningococcus 'phage have been found to lyse different strains completely in the following dilutions, viz., three strains in 1 in 1,000,000, three strains in 1 in 100,000, five strains in 1 in 10,000, six strains in 1 in 1,000, nine strains in 1 in 100, and all strains in 1 in 10.

6. Clinical results, however, tend to show that, *in vivo*, the meningococcus 'phage can grow and produce its effects when administered intravenously although subjected to temperatures at or above body temperature.

ACKNOWLEDGMENTS.

In conclusion, I record my sincere thanks to my assistants and colleagues, particularly Mr. S. Acharya, Dr. K. Guha and Dr. K. Ganguly, for working with me in this connection.

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STUDIES ON THE UTILITY OF RAMON'S FLOCCULATION TEST IN THE STANDARDIZATION OF TETANUS ANTITOXIN.

BY

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AFTER his preliminary discovery that flocculation occurs in a mixture of diphtheria toxin and antitoxin, Ramon (1922) introduced the method of standardization of diphtheria antitoxin *in vitro*, by mixing different dilutions of antitoxic serum with constant amounts of toxin and noting which tube in such a series first showed flocculation. Further work, confirmatory or contradictory, was directed towards the practical aspect of the problem, namely, whether the results of *in vitro* and *in vivo* tests were the same. Dean and Webb (1926) working with horse serum and anti-horse rabbit serum introduced another method for comparing the 'concentration' of antibody in different samples of anti-horse rabbit serum, where they used constant amounts of antibody and varying dilutions of antigen. They noted that the mixture showing the earliest flocculation did actually correspond to chemically equivalent amounts of antigen and antibody and the supernatant contained neither free antigen nor free antibody, showing that all of these had united with the formation of floccules. Hence their method was to ascertain the constant antibody optimal ratio. In comparison, Ramon's method was finding the constant antigen optimal ratio.

The work of Dean and Webb raised the question whether constant antigen optimal ratio and constant antibody optimal ratio corresponded with one another in all the systems and, if not, which of the ratios represented complete neutralization. Taylor (1933), Duncan (1932), and some other workers working with different systems, found that the two optimal ratios differed from one another to varying extents, and Duncan noted that the constant antigen optimal ratio corresponded to a mixture that contained a gross excess of antibody.

Those works naturally raised doubts about the accuracy of the method in the standardization of antitoxin, which required finding, firstly, the constant antibody optimal ratio, using constant amounts of standard antitoxin and varying amounts of test toxin; and, secondly, the constant antigen optimal ratio, using constant amounts of toxin containing certain L_+ doses as determined from the first procedure, and varying dilutions of unknown serum. If the two optimal ratios differed from one another, then the deductions based on them also became inaccurate. However,

the work of Miles (1933) showed that in the case of diphtheria toxin and antitoxin mixtures the two optimal ratios differed only very slightly. A number of workers also agreed that the results of *in vitro* and *in vivo* tests corresponded with one another quite closely and the method gained wide acceptance in the standardization of diphtheria antitoxin.

However, the application of the method in the standardization of tetanus antitoxin is not yet established beyond all controversy. Ramon and Descomby (1926) used the flocculation method for determining the potency of tetanus toxin and they claimed the same good result as in the case of diphtheria toxin, but only provided the serum used for the titration of tetanus toxin was 'highly suitable'. Abt and Erber (1926) applied the same technique, as used for the titration of diphtheria antitoxin, to the standardization of antitetanic serum. They found that the results of *in vitro* and *in vivo* tests did not correspond in all cases; nor in many cases did the serum flocculate regularly. Schmidt (1928) also corroborated the findings of Abt and Erber and noted that, in certain cases, there were two or more zones of initial flocculation which complicated the interpretation of the result.

Recently, Ramon, Lemetayer and Richou (1937) compared the antigenic power of tetanus toxin determined by the flocculation method, with its combining power and toxicity determined by animal experiment, and they found equivalent results in almost all cases. Ramon (1937), in another paper, also reported good results in the flocculation of a mixture of a recent and highly active filtrate of broth culture of *Cl. tetani* and a convenient corresponding antitoxin, and he believed that the mixture showing the 'flocculation initiale' represented the perfectly neutral mixture of toxin and antitoxin. He concluded that his 'flocculation initiale' method had precisely the same significance in the case of tetanus toxin and antitoxin mixture, as it had in the case of diphtheria toxin and antitoxin mixtures. In a recent paper, Ghosh and Ray (1937) reported that the potency of concentrated tetanus antitoxin determined by the flocculation test agreed well with that determined by experiment *in vivo*.

All these divergent reports about the practical utility of the test in the standardization of tetanus antitoxin naturally raises the question whether the flocculation method is at least theoretically sound in this particular case or not. The present series of experiments have been undertaken on this point.

EXPERIMENTAL.

The nature of the mechanism of the flocculation of a mixture of tetanus toxin and antitoxin was studied from three different aspects:—

- (i) The uniformity or otherwise in the rapidity of flocculation.
- (ii) The relation of the constant antibody to the constant antigen optimum proportions.
- (iii) Whether the mixture showing first flocculation represented the equivalent proportions of toxin and antitoxin or not.

The technique common to all the experiments was as follows:—

The sera used for the experiments were a mixture of samples collected from a number of horses (about 20 on the average) to minimize the effect of individual variations. They were collected the day before the experiment commenced, stored at

0°C. to 2°C. overnight and centrifuged before use to remove any suspended R. B. Cs. Their titre was determined by animal experiment simultaneously. The average titre was about 700 International Units per c.c.

The toxins used were also a mixture of several samples of veal-peptone-broth cultures of *Cl. tetani*, grown at 37°C. for 10 days and filtered through Seitz pads just before use. The lethal dose was on the average 1/20,000 c.c. for a guinea-pig of 350 g. weight, in 4 days: the pH was about 7.2.

The mixtures were put in $\frac{3}{4}$ -inch tubes in a water-bath at 45°C., so that two-thirds of the mixtures remained below the water level and one-third outside, in order to facilitate the constant agitation of the mixtures by the convection current.

The flocculation was observed in a box with a black background and mechanism for oblique illumination, where all the stages—commencing with slight to distinct opalescence and the formation of minute floccules moving with the convection current (if the reaction did not stop at the limit of opalescence only) and ending in the formation of cotton-wool-like flakes—could be followed distinctly. The formation of distinct opalescence was taken as the end-point, as in some experiments floccules did not form at all.

Where more than eight hours' incubation was necessary, the mixtures were put in the ice-chest at 0°C. to 2°C. overnight and incubated again next day. The experiments were concluded after a total of 15 hours' incubation in the water-bath, after which no further reading was taken.

I. *The uniformity or otherwise in the rapidity of flocculation of mixtures of tetanus toxin and antitoxin in varying proportions.*

To 20 tubes, each containing 10 c.c. of toxin broth, 1.0 c.c. to 0.005 c.c. of anti-serum was added successively and the total volume in each tube was made up to 11 c.c. with normal saline. The tubes were then placed in the water-bath and the time taken for the occurrence of distinct opalescence in each tube was noted. The result is shown in Table I:—

TABLE I.

Tube number.	Amount in c.c. of serum added to 10 c.c. toxin.	Time in hours.	Tube number.	Amount in c.c. of serum added to 10 c.c. toxin.	Time in hours.
1	1.0	..	11	0.09	..
2	0.9	..	12	0.08	..
3	0.8	..	13	0.07	9
4	0.7	14	14	0.06	7
5	0.6	10	15	0.05	6
6	0.5	8	16	0.04	10½
7	0.4	9	17	0.03	11
8	0.3	11	18	0.02	12
9	0.2	3	19	0.01	12½
10	0.1	..	20	0.005	14½

Hence the ratio of the constant antibody to the constant antigen optimum proportion was 1.8. The ratio determined from the data of the previous series was practically the same, viz., 1.9.

III. *Whether the mixture showing earliest flocculation represented the equivalent proportion of the toxin and antitoxin or not.*

This could only be determined by finding whether all the toxin and antitoxin molecules had been carried down with the floccules, or some of either or both was left behind in the supernatant. Hence the procedure consisted in centrifuging down the coarse floccules and examining the clear supernatant for the presence of free toxin and antitoxin. But the technical difficulty arose in the detection of free toxin. This could be done firstly by animal experiment to demonstrate toxicity, and secondly by its flocculation with antitoxin. The first procedure was useless as prolonged incubation at 45°C. in the presence of air, diminished the toxicity to such an extent as to render its detection, when present in small amounts, impossible. The second procedure was also of no use, as the range of flocculability of the toxin and antitoxin was so narrow as to render impossible the detection of small amounts left in the supernatant in a state of high dilution. So, the only thing that could be done without recourse to special technique, was to detect the presence of free antitoxin in the supernatant, by animal experiment.

For this purpose, 1 c.c. of the supernatants of the centrifuged flocculated mixtures representing the constant antibody and the constant antigen optimum proportions respectively, as determined in the previous experiments, was mixed with 1 c.c. of toxin containing one L_+ dose, incubated at 37°C. for 30 minutes, and the whole amount was injected subcutaneously in two guinea-pigs of 350 g. weight, for each supernatant. The result is given in Table IV:—

TABLE IV.

Guinea-pig number.	Nature of inoculum.	Result.
1	Supernatant of constant antibody optimal tube + one L_+ dose of toxin.	Living after four days.
2	Do.	Do.
3	Supernatant of constant antigen optimal tube + one L_+ dose of toxin.	Do.
4	Do.	Do.

The result of this experiment was very striking. Leaving aside the consideration of the constant antigen optimum proportion, which required about double the amount of antibody as required for the constant antibody optimum proportion,

the result of the titration of the supernatant of the constant antibody optimum proportion mixture was definite. As the sample of serum used for the experiment contained about 700 I. U. per c.c., the original content of each c.c. of the mixture showing constant antibody optimum proportion was about 22·5 I. U., of which at least one I. U. was left behind after flocculation. Hence at least $1/22\cdot5$ part of antitoxin did not enter into the composition of the floccules.

DISCUSSION.

The conclusions based on the results of the experiments do not speak highly for the theoretical accuracy of the test in the standardization of tetanus antitoxin. This can possibly be explained by the assumption that the toxin used for the production of antibody and in carrying out the *in vitro* tests is very highly complex. It is possibly a mixture of a number of antigens producing varying types of antibodies to a varying degree, together with the tetanus antitoxin. The reaction *in vitro* of these multiple antigens with varying amounts of multiple antibodies as also other non-specific proteins in the horse serum, might be responsible for the discrepancies, which are not observed with comparatively simpler antigens and antibodies. Whereas with *in vivo* tests, the presence of only the tetanus toxin and the antitoxin molecules are detected, the other antigenic components having no noticeable affinity for the animal tissues and the corresponding antibodies being also useless.

The antigenic constitution of the diphtheria toxin is perhaps simpler. The changes occurring in the broth as a result of the growth of the organisms determine the nature of the toxin, and that produced by the growth of *C. diphtheriæ* may possibly be simpler antigenically than the changes produced by the growth of *Cl. tetani*. This may explain the comparative accuracy of the method in the titration of diphtheria antitoxin. These explanations are hard to prove till the toxin can be obtained in a pure state, the corresponding antitoxin be produced with it, and the tests repeated with the pure toxin and the corresponding antitoxin.

As found by many observers this method may be empirically useful when the results of the *in vitro* and the *in vivo* tests correspond. The results, however, frequently fail to correspond, especially when there are more than one zone of initial flocculation. Hence this method cannot be fully relied upon, even empirically, for the standardization of tetanus antitoxin.

SUMMARY.

The theoretical accuracy of Ramon's method of standardization of the tetanus antitoxin was investigated. It was found that:—

1. There was no uniformity in the rapidity of flocculation of mixtures of tetanus toxin and antitoxin in varying proportions.
2. The ratio of the constant antibody to the constant antigen optimum proportion was about 1·8.
3. The supernatants of the centrifuged flocculated mixtures of the constant antibody and the constant antigen optimum proportions showed clear evidence of the presence of antitoxin so that those optima did not represent perfectly neutral points.

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NUMERICAL ESTIMATION OF *B. COLI* BY DILUTION METHOD.

BY

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THE accepted method of bacteriological examination of water to test its purity for the purpose of determining its potability is to estimate the number of *B. coli* present in a given volume. For given sources of supplies standards have been, more or less arbitrarily, established to which water must conform before it is passed fit for drinking purposes. The usual way of expressing the final result is to state the presence or absence of *B. coli* in 100 c.c., 50 c.c., or smaller quantities of water. Till recently the samples of water of quantities mentioned above were inoculated in liquid media and the tubes showing evidence of fermentation with gas formation were taken to contain *B. coli*. Since, however, a single organism may give the same evidence of fermentation as a larger number might do this method was inadequate for quantitative estimation. It was also fallacious due to the fact that owing to random distribution of the organisms in the water a smaller quantity of the sample might give a positive result while a larger quantity might fail to do so. In any case, there was no satisfactory way of eliminating the discordant results even when a number of tubes were inoculated with equal quantities of water. Thus, by this method two observers testing water from the same source might get different results and pass contradictory judgments on the quality of water. Thresh, Beale and Suckling (1933) recommended that both the smallest quantity of water from which *B. coli* was isolated and the largest quantity from which it was not should be stated. This procedure is also open to the same objections. To make the point clear their example may be given: 'Thus: supposing a positive result was obtained in the 1 c.c. tubes and a negative in the 0.1 c.c. tubes then the findings are recorded as *B. coli* present in 1 c.c., absent in 0.1 c.c. or as over 100 but less than 1,000 *B. coli* per 100 c.c. of water'. It may be noted that they have in the above statement suggested a method for the assessment of the probable number of bacteria in a unit volume. Greenwood and Yule (1917) quote Beveridge and Wanhill (1912) who provide another method which consists in taking a number of samples of the same volume and calculating the probable number of *B. coli* from the proportion of tubes giving positive results. For example, 'if 10 tubes each inoculated with 1 c.c. of water yield 3 positive and 7 negative results the sample may be considered to contain

B. coli in every 3 c.c. of water'. This method, the authors said, was only a rough approximation. It may be noted that Beveridge and Wanhill's method is an advance on others inasmuch as it takes into consideration results of a number of identical experiments. It also clearly brings out the fact that if a given volume of water fails to give evidence of the presence of *B. coli* it in no wise proves its sterility with regard to *B. coli*. The criticism of this method of estimation has been expressed in clear terms by the Ministry of Health (1934) as follows: 'parallel tests on the same sample of water may give different results, e.g., the smallest quantity giving a positive reaction may be 10 ml. in the first experiment, 50 ml. in the second, and 1 ml. in the third, whilst a fourth test may give a positive reaction in 1 ml. and a negative in 10 ml. This seemingly anomalous behaviour is due mainly to the fact that the test is one of random sampling, the positive and negative results depending on the chance distribution of viable *coli-aerogenes* bacteria in the water. It had frequently been the cause of differences, both in observation and in interpretation, between different analysts working on the same water'. The need therefore arose for the standardization of the methods and the development of the mathematical theory for this purpose.

McCrary (1915), Greenwood and Yule (*loc. cit.*), Wolman and Weaver (1917), and Stein (1919) have studied the problem and made important contributions towards the development of the statistical theory for this purpose. Without entering into detailed consideration of the methods devised by them the general principles on which they are based may be stated as follows:—

If m be the mean number of organisms present in a unit volume of water and if we take a number of samples of unit size then the distribution of these samples with regard to the number of organisms contained in each will be as given in Table I:—

TABLE I.
*Distribution of the samples with different numbers
of bacteria.*

Number of bacteria.	Proportion of samples of unit volume containing stated number of bacteria.
0	e^{-m}
1	$e^{-m} \cdot m$
2	$e^{-m} \cdot \frac{m^2}{2!}$
3	$e^{-m} \cdot \frac{m^3}{3!}$
.	.
.	.
.	.
x	$e^{-m} \cdot \frac{m^x}{x!}$
.	.
.	.
.	.

In other words, the frequency distribution of samples containing 0, 1, 2,x, organisms in the unit volume follows a Poisson's series.

In the report of the Ministry of Health (*loc. cit.*), it is stated that the above distribution and the calculations entering in the construction of the tables for probable number of organism are based in a quantitative sense on the assumption that the smallest quantity of water giving *coli*-reaction contained one viable bacillus'. This assumption does not appear to be necessary in formulating the above frequency distributions. All that is necessary, as Fisher (1934) has said, is to ensure that the technique of dilution afforded a perfectly random distribution of organisms, and that these could develop . . . without mutual interference'.

In recent years tables have been constructed for the calculation of the probable number of organisms which could be estimated from the results of parallel tube experiments. The calculations entering into the construction of these tables are based on formulæ devised by Greenwood and Yule (*loc. cit.*) as modified by McCrady (1918). The Ministry of Health have adopted these tables for certain sets of dilutions for purposes of general reference.

Fisher (1921) in the course of exemplifying the applicability of his 'method of maximum likelihood' has attacked the same problem. He has given formulæ for the calculation of the logarithm of the most probable number and also for the estimation of the standard error for this logarithm. His work is important in that it supplies a perfectly general and yet a simpler method for the development of necessary formulæ. It further differs from the method of approach of Greenwood and Yule in an important respect. These authors base their formulæ on the assumption of Baye's theorem. Fisher has made no such assumption and he has further shown that this assumption in inductive reasonings of this type may sometimes lead to erroneous conclusions.

Halvorson and Ziegler (1932) have adopted the process suggested by Fisher and have obtained practically the same formulæ as could be deduced from the expression which Greenwood and Yule have given. We may here point out that the same formula had already been put out by Fisher in 1921. Fisher's formula for the most probable value of n reduces to the form:—

$$S \left[-\frac{1}{a^x} \left(\frac{v_x \cdot e^{-n/a^x}}{1 - e^{-n/a^x}} - u_x \right) \right] = 0 \dots\dots\dots (1)$$

where 'a' denotes the dilution factor, x the stage of the dilution, v_x is the number of tubes found positive and u_x those found negative when working with the a^x th dilution and 'e' is the usual exponential base. 'S' denotes summation over all the dilutions. Halvorson and Ziegler realized that this equation could not be solved in general but a solution could be obtained by trial and error. For this purpose they have put it in a modified form and have provided tables of $\frac{1}{1 - e^{-x}}$ for various values of x.

We may, however, put this formulæ in a slightly different form as follows:—

$$S \left[\frac{1}{a^x} \left(\frac{v_x}{e^{n/a^x} - 1} - u_x \right) \right] = 0 \dots\dots\dots$$

OR
$$S \left(\frac{v_x}{a^x} \frac{E_x - u_x}{1} \right) = 0 \text{ where } E_x = \frac{1}{e^{n/a^x} - 1} \dots\dots\dots (2)$$

We shall refer to this simplification later on.

As stated previously Fisher (1921) had also provided a method for getting a formula for the standard errors of the most probable numbers calculated by the above formula. Using Fisher's method we have tried to develop a formula for the standard error of the most probable number.

If p_x and q_x are the probabilities of getting sterile and fertile samples at the a^x th dilution then $p_x = e^{-n/a^x}$ and $q_x = 1 - p_x$.

The chance P of getting the combination such as v_x fertile and u_x sterile where x embraces all dilutions can be expressed as

$P = \pi k_x p_x^{u_x} q_x^{v_x}$ where π denotes the products of various p 's and q 's raised to appropriate indices which depend upon the results of different dilutions and k_x is a constant.

$\log P = K + S \left[u_x \log p_x + v_x \log q_x \right] = L$ (say). Differentiating this twice with regard to n we get

$$\frac{d^2 L}{dn^2} = -S \left[\frac{1}{a^{2x}} \cdot v_x \left(\frac{e^{n/a^x}}{e^{n/a^x} - 1} \right)^2 \right].$$

The mean value of this expression when s_x tubes are taken for the a^x th dilution is

$$-S \left[\frac{s_x}{a^{2x}} \cdot \frac{p_x}{q_x} \right] = -S \left[\frac{s_x E_x}{a^{2x}} \right].$$

If σ_n is the standard error of n we therefore have

$$\frac{1}{\sigma_n^2} = S \left(\frac{s_x E_x}{a^{2x}} \right) \dots\dots\dots (3)$$

and if the same number of tubes s are used in each dilution the formulæ for the standard error of n becomes

$$\frac{1}{\sigma_n^2} = s S \left(\frac{E_x}{a^{2x}} \right) \dots\dots\dots (4)$$

A perusal of the formulæ (2), (3), and (4) will make it clear that of the symbols entering into the formula u_x , v_x , i.e., the numbers of sterile and

fertile samples are known from the results of the experiments and a^x is the corresponding dilution. The only other function that enters in the formulæ for the calculation of n and of the standard error of n is E_x . If, therefore, this function can be tabulated for various values of n , the solution of both types of formulæ by trial and error will be simplified. These values have now been calculated and are presented in the *Appendix* which gives corresponding to a value of n the corresponding values of $E_{n/10}$, $E_{n/100}$, $E_{n/1,000}$. The *Appendix* is given in a form which is most convenient for use when the dilution factor is 10 and three dilutions are taken. It may, however, be seen that the *Appendix* is of a much wider application because whatever be the dilution the appropriate value of E_x may still be read from it.

An example will perhaps make the use of the formulæ clear. Let us say that five tubes for each of the dilutions 1/10, 1/100 and 1/1,000 are taken and the tubes which are positive for each of these dilutions are 3, 2, and 1, respectively.

$$\begin{array}{ll} \text{We therefore have } a &= 10, & x &= 1, 2 \text{ or } 3, \\ &u_1 &= 2, &v_1 &= 3, \\ &u_2 &= 3, &v_2 &= 2, \\ &u_3 &= 4, &v_3 &= 1. \end{array}$$

$$\begin{aligned} \text{Substituting these values in the equation (1) we have } &\frac{1}{10} (3.E_{n/10} - 2) + \\ &\frac{1}{100} (2.E_{n/100} - 3) + \frac{1}{1,000} (E_{n/1,000} - 4) = 0 \\ \text{or } 300 E_{n/10} + 20 E_{n/100} + E_{n/1,000} &= 234. \end{aligned}$$

$$\text{For } n = 18 \text{ left hand side} = 215.88$$

$$n = 17 \quad \text{,,} \quad \text{,,} \quad \text{,,} = 233.31$$

$$n = 16 \quad \text{,,} \quad \text{,,} \quad \text{,,} = 253.16$$

The value 17 gives the nearest value to the right hand side and hence is the solution of the equation. Therefore, the most probable number of organisms in the sample tested is 17.

The next problem is to find the standard error of 17. Since the number of tubes is constant we make use of formula (4).

$$\frac{1}{\sigma_n^2} = 5 \left[\frac{E_{17/10}}{10^2} + \frac{E_{17/100}}{10^4} + \frac{E_{17/1,000}}{10^6} \right].$$

The values of the three E 's corresponding to 17 can be read from the tables and we have

$$\frac{1}{\sigma_n^2} = 5 \left[.002235 + .000540 + .000058 \right]$$

$$\text{or } n = \pm 8.$$

We may now examine the probable values as shown by various combinations of positive and negative samples which have been computed by McCrady (1918) and have been adopted by the Ministry of Health (*loc. cit.*).

The first table relates to the case when for the three dilutions $\frac{1}{2}$, $\frac{1}{10}$, and $\frac{1}{100}$ we use 1, 5, and 5 tubes, respectively. The second relates to the case when the dilutions are $\frac{1}{10}$, $\frac{1}{100}$, and $\frac{1}{1,000}$ and five tubes are used in each case. Our table gives the same probable values up to $n = 20$ except in two cases and this is perhaps due to a mistake in his computation. For the probable numbers exceeding 20, McCrady's tables give only approximate values. This approximation has now been removed and all the values now given are correct to unit place.

TABLE II.

Showing the most probable number of bacteria present in 100 c.c. of water and the standard error of this number, for various combinations of positive and negative results when 1 tube is used for 50 c.c., 5 tubes are used for 10 c.c., and 5 tubes are used for 1 c.c.

NUMBER OF TUBES GIVING POSITIVE REACTION.			Most probable number of organisms in 10 c.c.	Standard error of the most probable number.
50 c.c. 1 tube used.	10 c.c. 5 tubes used.	1 c.c. 5 tubes used.		
0	0	1	1	1
0	0	2	2	2
0	1	0	1	1
0	1	1	2	2
0	1	2	3	2
0	2	0	2	2
0	2	1	3	2
0	2	2	4	3
0	3	0	3	2
0	3	1	5	3
0	4	0	5	3
1	0	0	1	1
1	0	1	3	2
1	0	2	4	3
1	0	3	6	3

TABLE II—*concl.*

NUMBER OF TUBES GIVING POSITIVE REACTION.			Most probable number of organisms in 10 c.c.	Standard error of the most probable number.
50 c.c. 1 tube used.	10 c.c. 5 tubes used.	1 c.c. 5 tubes used.		
1	1	0	3	2
1	1	1	5	3
1	1	2	7	4
1	1	3	9	5
1	2	0	5	3
1	2	1	7	4
1	2	2	10	5
1	2	3	12	6
1	3	0	8	4
1	3	1	11	6
1	3	2	14	7
1	3	3	18	9
1	3	4	21	10
1	4	0	13	7
1	4	1	17	8
1	4	2	22	11
1	4	3	28	14
1	4	4	35	19
1	4	5	43	25
1	5	0	24	12
1	5	1	35	19
1	5	2	54	33
1	5	3	92	55
1	5	4	161	89

TABLE III.

Showing the most probable number of bacteria present in 100 c.c. of water and the standard error of this number for various combinations of positive and negative results when 5 tubes are used for each of the quantities 10 c.c., 1 c.c., and 0.1 c.c.

NUMBER OF TUBES GIVING POSITIVE REACTION.			Most probable number of organisms in 100 c.c.	Standard error of the most probable number.
10 c.c. 5 tubes used.	1 c.c. 5 tubes used.	0.1 c.c. 5 tubes used.		
0	0	1	2	2
0	0	2	4	3
0	1	0	2	2
0	1	1	4	3
0	1	2	6	4
0	2	0	4	3
0	2	1	6	4
0	3	0	6	4
1	0	0	2	2
1	0	1	4	3
1	0	2	6	4
1	0	3	8	5
1	1	0	4	3
1	1	1	6	4
1	1	2	8	5
1	2	0	6	4
1	2	1	8	5
1	2	2	10	5
1	3	0	8	5
1	3	1	10	5
1	4	0	11	6
2	0	0	5	3

TABLE III—*contd.*

NUMBER OF TUBES GIVING POSITIVE REACTION.			Most probable number of organisms in 100 c.c.	Standard error of the most probable number.
10 c.c. 5 tubes used.	1 c.c. 5 tubes used.	0.1 c.c. 5 tubes used.		
2	0	1	7	4
2	0	2	9	5
2	0	3	12	6
2	1	0	7	4
2	1	1	9	5
2	1	2	12	6
2	2	0	9	5
2	2	1	12	6
2	2	2	14	7
2	3	0	12	6
2	3	1	14	7
2	4	0	15	8
3	0	0	8	5
3	0	1	11	6
3	0	2	13	7
3	1	0	11	6
3	1	1	14	7
3	1	2	17	8
3	1	3	20	10
3	2	0	14	7
3	2	1	17	8
3	2	2	20	10
3	3	0	17	8
3	3	1	21	10
3	4	0	21	10
3	4	1	24	12

TABLE III--*contd.*

NUMBER OF TUBES GIVING POSITIVE REACTION.			Most probable number of organisms in 100 c.c.	Standard error of the most probable number.
10 c.c. 5 tubes used.	1 c.c. 5 tubes used.	0.1 c.c. 5 tubes used.		
3	5	0	25	12
4	0	0	13	7
4	0	1	17	8
4	0	2	21	10
4	0	3	25	12
4	1	0	17	8
4	1	1	21	10
4	1	2	26	13
4	2	0	22	11
4	2	1	26	13
4	2	2	32	17
4	3	0	27	14
4	3	1	33	17
4	3	2	39	21
4	4	0	34	18
4	4	1	40	22
4	5	0	41	23
4	5	1	48	27
5	0	0	23	11
5	0	1	31	16
5	0	2	43	24
5	0	3	58	34
5	0	4	76	44
5	1	0	33	17
5	1	1	46	26
5	1	2	63	37

TABLE III—*concl'd.*

NUMBER OF TUBES GIVING POSITIVE REACTION.			Most probable number of organisms in 100 c.c.	Standard error of the most probable number.
10 c.c. 5 tubes used.	1 c.c. 5 tubes used.	0.1 c.c. 5 tubes used.		
5	1	3	84	47
5	2	0	49	28
5	2	1	70	41
5	2	2	94	52
5	2	3	120	63
5	2	4	148	75
5	2	5	177	88
5	3	0	79	45
5	3	1	109	58
5	3	2	141	72
5	3	3	175	87
5	3	4	212	106
5	3	5	253	128
5	4	0	130	67
5	4	1	172	86
5	4	2	221	111
5	4	3	278	143
5	4	4	345	187
5	4	5	426	246
5	5	0	240	121
5	5	1	348	189
5	5	2	542	330

The standard errors of these probable numbers have also been calculated and are given in Tables II and III along with the probable numbers.

EFFICIENCY OF THE DILUTION METHOD.

A perusal of Table III will show that the standard error attached to each probable number is much too large, being about one-half of the number itself. Formula (4) which gives the standard error involves E_x , s_x and a^2x ; in other words, the standard error depends upon the probable number, the dilution and the number of the tubes used. For practical purposes it would be of interest to find out how many tubes and what dilutions it is necessary to use to increase the accuracy of the probable number to a reasonable extent. We propose to discuss the question of dilution in another communication, but the problem relating to the increasing of the number of tubes is quite simple and may be examined here.

Since σ_n varies with $\sqrt{s_x}$ for any given probable number the dilution remaining constant, we can reduce σ_n to any desired extent by increasing s_x . If, for instance, it is desired to reduce σ_n so that it is nearly one-tenth of the probable number we shall have to increase $\sqrt{s_x}$ about five times, i.e., the number of tubes to be used will have to be increased 25 times, or 125 tubes instead of 5 will have to be used. From the relation $\sigma_n = \frac{c}{\sqrt{s_x}}$ it is easy to calculate roughly the number of tubes to be used in order to increase the accuracy to any desired extent.

SUMMARY.

The problem of the estimation of most probable number of organisms present in a water supply as tested by the inoculation of varying quantities in a liquid medium is discussed. A suitable method for the determination of the standard error attached to the probable number has been worked out and tables have been constructed which could be used to simplify calculation for the determination of the most probable number and the standard error of this number. The relationship between the number of tubes used for any set of dilutions and the accuracy of the probable number is given. The tables for the probable numbers given by the Ministry of Health have been reconstructed so as to give the value of the probable numbers correct to the nearest unit and their standard errors have also been calculated.

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APPENDIX.

Values of $\frac{1}{e^{\frac{n}{x}} - 1}$ for values of n from 1 to 1,000 when x is equal to 1, 2, or 3.

n	$\frac{1}{e^{\frac{n}{10}} - 1}$	$\frac{1}{e^{\frac{n}{100}} - 1}$	$\frac{1}{e^{\frac{n}{1,000}} - 1}$	n	$\frac{1}{e^{\frac{n}{10}} - 1}$	$\frac{1}{e^{\frac{n}{100}} - 1}$	$\frac{1}{e^{\frac{n}{1,000}} - 1}$
1	9.508332	99.500833	999.500083	24	0.099769	3.686647	41.168667
2	4.516656	49.501667	499.500167	25	0.089425	3.520812	39.502083
3	2.858296	32.835833	332.833583	26	0.080233	3.367796	37.963705
4	2.033245	24.503333	249.500333	27	0.072048	3.226176	36.539287
5	1.541494	19.504166	199.500417	28	0.064747	3.094731	35.216619
6	1.216369	16.171666	166.167167	29	0.058227	2.972409	33.985175
7	0.986434	13.791547	142.357726	30	0.052396	2.858296	32.835833
8	0.815966	12.006666	124.500667	31	0.047174	2.751599	31.760648
9	0.685118	10.618610	110.611861	32	0.042494	2.651621	30.752667
10	0.581977	9.508332	99.500833	33	0.038296	2.557753	29.805780
11	0.498961	8.600074	90.410008	34	0.034525	2.469455	28.914598
12	0.431013	7.843331	82.834333	35	0.031138	2.386250	28.074345
13	0.374631	7.203138	76.424160	36	0.028091	2.307713	27.280778
14	0.327311	6.654520	70.929738	37	0.025350	2.233466	26.530110
15	0.287217	6.179162	66.167917	38	0.022883	2.163170	25.818956
16	0.252970	5.763328	62.001333	39	0.020660	2.096520	25.144276
17	0.223516	5.396513	58.324946	40	0.018657	2.033245	24.503333
18	0.198034	5.070547	55.057036	41	0.016852	1.973096	23.893660
19	0.175874	4.778982	52.133162	42	0.015224	1.915850	23.313024
20	0.156518	4.516656	49.501667	43	0.013755	1.861305	22.759397
21	0.139545	4.279392	47.120798	44	0.012430	1.809276	22.230939
22	0.124610	4.063773	44.956379	45	0.011234	1.759596	21.725972
23	0.111431	3.866976	42.980178	46	0.010154	1.712112	21.242964

APPENDIX—contd.

n	$\frac{1}{e^{n/10} - 1}$	$\frac{1}{e^{n/100} - 1}$	$\frac{1}{e^{n/1,000} - 1}$	n	$\frac{1}{e^{n/10} - 1}$	$\frac{1}{e^{n/100} - 1}$	$\frac{1}{e^{n/1,000} - 1}$
47	0.009179	1.666683	20.780512	73	0.000676	0.930163	13.204713
48	0.008298	1.623181	20.337333	74	0.000612	0.912462	13.019680
49	0.007502	1.581487	19.912246	75	0.000553	0.895255	12.839583
50	0.006784	1.541494	19.504166	76	0.000501	0.878521	12.664227
51	0.006134	1.503101	19.112093	77	0.000453	0.862243	12.493429
52	0.005547	1.466216	18.735102	78	0.000410	0.846402	12.327012
53	0.005017	1.430754	18.372341	79	0.000371	0.830981	12.164810
54	0.004537	1.396635	18.023018	80	0.000336	0.815966	12.006666
55	0.004104	1.363786	17.686401	81	0.000304	0.801341	11.852428
56	0.003712	1.332139	17.361809	82	0.000275	0.787092	11.701955
57	0.003357	1.301631	17.048609	83	0.000249	0.773205	11.555109
58	0.003037	1.272202	16.746212	84	0.000225	0.759667	11.411761
59	0.002747	1.243799	16.454069	85	0.000204	0.746465	11.271788
60	0.002485	1.216369	16.171666	86	0.000184	0.733589	11.135073
61	0.002248	1.189865	15.898526	87	0.000167	0.721027	11.001502
62	0.002034	1.164242	15.634199	88	0.000151	0.708768	10.870969
63	0.001840	1.139458	15.378266	89	0.000136	0.696801	10.743371
64	0.001664	1.115473	15.130333	90	0.000123	0.685118	10.618610
65	0.001506	1.092251	14.890032	91	0.000112	0.673708	10.496593
66	0.001362	1.069756	14.657015	92	0.000101	0.662563	10.377231
67	0.001232	1.047957	14.430956	93	0.000091	0.651674	10.260437
68	0.001115	1.026823	14.211549	94	0.000083	0.641033	10.146130
69	0.001009	1.006324	13.998503	95	0.000075	0.630632	10.034231
70	0.000913	0.986434	13.791547	96	0.000068	0.620464	9.924665
71	0.000826	0.967126	13.590423	97	0.000061	0.610521	9.817360
72	0.000747	0.948377	13.394888	98	0.000055	0.600797	9.712247

APPENDIX—contd.

n	$\frac{1}{e^{n/10} - 1}$	$\frac{1}{e^{n/100} - 1}$	$\frac{1}{e^{n/1,000} - 1}$	n	$\frac{1}{e^{n/10} - 1}$	$\frac{1}{e^{n/100} - 1}$	$\frac{1}{e^{n/1,000} - 1}$
99	0.000050	0.591284	9.609259	125	0.000001	0.401551	7.510414
100	0.000045	0.581977	9.508332	126	0.000003	0.395974	7.447005
101	0.000041	0.572869	9.409405	127	0.000003	0.390495	7.384596
102	0.000037	0.563954	9.312420	128	0.000003	0.385113	7.323164
103	0.000034	0.555227	9.217320	129	0.000002	0.379826	7.262685
104	0.000030	0.546682	9.124050	130	0.000002	0.374631	7.203138
105	0.000028	0.538314	9.032558	131	0.000002	0.369525	7.144501
106	0.000025	0.530118	8.942794	132	0.000002	0.364508	7.086754
107	0.000023	0.522090	8.854709	133	0.000002	0.359577	7.029877
108	0.000020	0.514224	8.768258	134	0.000002	0.354730	6.973850
109	0.000018	0.506515	8.683394	135	0.000001	0.349965	6.918654
110	0.000017	0.498961	8.600074	136	0.000001	0.345281	6.864271
111	0.000015	0.491555	8.518257	137	0.000001	0.340675	6.810683
112	0.000014	0.484296	8.437903	138	0.000001	0.336146	6.757873
113	0.000012	0.477177	8.358972	139	0.000001	0.331691	6.705824
114	0.000011	0.470197	8.281428	140	0.000001	0.327311	6.654520
115	0.000010	0.463351	8.205233	141	0.000001	0.323002	6.603945
116	0.000009	0.456635	8.130354	142	0.000001	0.318764	6.554083
117	0.000008	0.450046	8.056756	143	0.000001	0.314594	6.504920
118	0.000008	0.443582	7.984407	144	0.000001	0.310492	6.456440
119	0.000007	0.437239	7.913276	145	0.000001	0.306456	6.408631
120	0.000006	0.431013	7.843331	146	0.000000	0.302484	6.361477
121	0.000006	0.424902	7.774544	147	0.000000	0.298576	6.314967
122	0.000005	0.418903	7.706885	148	0.000000	0.294729	6.269086
123	0.000005	0.413013	7.640329	149	0.000000	0.290943	6.223821
124	0.000004	0.407230	7.574847	150	0.000000	0.287217	6.179162

APPENDIX—contd.

n	$\frac{1}{\sigma^n/100 - 1}$	$\frac{1}{\sigma^n/1,000 - 1}$	n	$\frac{1}{\sigma^n/100 - 1}$	$\frac{1}{\sigma^n/1,000 - 1}$
151	0.283549	6.135095	179	0.200423	5.101501
152	0.279938	6.091609	180	0.198034	5.070547
153	0.276382	6.048693	181	0.195678	5.039937
154	0.272882	6.006335	182	0.193354	5.009664
155	0.269435	5.964524	183	0.191063	4.979722
156	0.266041	5.923251	184	0.188803	4.950107
157	0.262698	5.882505	185	0.186573	4.920813
158	0.259406	5.842275	186	0.184375	4.891835
159	0.256164	5.802553	187	0.182206	4.863168
160	0.252970	5.763328	188	0.180066	4.834806
161	0.249824	5.724591	189	0.177956	4.806746
162	0.246725	5.686334	190	0.175874	4.778982
163	0.243672	5.648547	191	0.173820	4.751509
164	0.240664	5.611222	192	0.171793	4.724324
165	0.237700	5.574350	193	0.169793	4.697421
166	0.234780	5.537923	194	0.167820	4.670796
167	0.231902	5.501934	195	0.165874	4.644445
168	0.229066	5.466374	196	0.163953	4.618364
169	0.226271	5.431236	197	0.162057	4.592548
170	0.223516	5.396513	198	0.160186	4.566994
171	0.220801	5.362196	199	0.158340	4.541698
172	0.218125	5.328280	200	0.156518	4.516656
173	0.215487	5.294756	201	0.154719	4.491863
174	0.212886	5.261619	202	0.152944	4.467317
175	0.210323	5.228862	203	0.151192	4.443013
176	0.207795	5.196477	204	0.149463	4.418949
177	0.205303	5.164460	205	0.147756	4.395120
178	0.202846	5.132803	206	0.146071	4.371523

APPENDIX—contd.

n	$\frac{1}{e^{n/100} - 1}$	$\frac{1}{e^{n/1,000} - 1}$	n	$\frac{1}{e^{n/100} - 1}$	$\frac{1}{e^{n/1,000} - 1}$
207	0.144408	4.348156	235	0.105423	3.774884
208	0.142766	4.325013	236	0.104265	3.756937
209	0.141145	4.302093	237	0.103121	3.739141
210	0.139545	4.279392	238	0.101990	3.721495
211	0.137965	4.256907	239	0.100873	3.703998
212	0.136404	4.234635	240	0.099769	3.686647
213	0.134864	4.212572	241	0.098678	3.669442
214	0.133343	4.190717	242	0.097600	3.652378
215	0.131842	4.169066	243	0.096536	3.635456
216	0.130359	4.147616	244	0.095483	3.618674
217	0.128894	4.126364	245	0.094443	3.602029
218	0.127449	4.105308	246	0.093416	3.585520
219	0.126021	4.084445	247	0.092401	3.569145
220	0.124610	4.063773	248	0.091397	3.552904
221	0.123218	4.043289	249	0.090405	3.536793
222	0.121842	4.022989	250	0.089425	3.520812
223	0.120484	4.002873	251	0.088457	3.504958
224	0.119142	3.982937	252	0.087500	3.489232
225	0.117817	3.963179	253	0.086554	3.473630
226	0.116508	3.943596	254	0.085619	3.458152
227	0.115215	3.924187	255	0.084695	3.442796
228	0.113938	3.904948	256	0.083781	3.427560
229	0.112677	3.885879	257	0.082879	3.412443
230	0.111431	3.866976	258	0.081986	3.397445
231	0.110200	3.848237	259	0.081105	3.382563
232	0.108984	3.829661	260	0.080233	3.367796
233	0.107783	3.811245	261	0.079371	3.353143
234	0.106596	3.792987	262	0.078519	3.338602

APPENDIX—contd.

n	$\frac{1}{e^{n/100} - 1}$	$\frac{1}{e^{n/1,000} - 1}$	n	$\frac{1}{e^{n/100} - 1}$	$\frac{1}{e^{n/1,000} - 1}$
263	0.077677	3.324173	291	0.057614	2.960642
264	0.076845	3.309853	292	0.057008	2.948956
265	0.076022	3.295642	293	0.056409	2.937351
266	0.075209	3.281539	294	0.055817	2.925825
267	0.074405	3.267542	295	0.055230	2.914378
268	0.073610	3.253650	296	0.054651	2.903009
269	0.072824	3.239862	297	0.054078	2.891717
270	0.072048	3.226176	298	0.053511	2.880501
271	0.071280	3.212593	299	0.052950	2.869361
272	0.070520	3.199109	300	0.052396	2.858296
273	0.069770	3.185725	301	0.051847	2.847305
274	0.069027	3.172440	302	0.051305	2.836387
275	0.068294	3.159251	303	0.050769	2.825541
276	0.067568	3.146159	304	0.050238	2.814768
277	0.066851	3.133162	305	0.049713	2.804066
278	0.066142	3.120259	306	0.049194	2.793434
279	0.065441	3.107449	307	0.048681	2.782872
280	0.064747	3.094731	308	0.048173	2.772379
281	0.064062	3.082105	309	0.047671	2.761955
282	0.063384	3.069568	310	0.047174	2.751599
283	0.062714	3.057121	311	0.046683	2.741309
284	0.062051	3.044762	312	0.046197	2.731086
285	0.061396	3.032490	313	0.045716	2.720929
286	0.060748	3.020304	314	0.045241	2.710837
287	0.060107	3.008204	315	0.044771	2.700810
288	0.059473	2.996189	316	0.044305	2.690847
289	0.058847	2.984257	317	0.043845	2.680947
290	0.058227	2.972409	318	0.043390	2.671110

APPENDIX—contd.

n	$\frac{1}{e^{n/100} - 1}$	$\frac{1}{e^{n/1,000} - 1}$	n	$\frac{1}{e^{n/100} - 1}$	$\frac{1}{e^{n/1,000} - 1}$
319	0.042940	2.661335	347	0.032116	2.410703
320	0.042494	2.651621	348	0.031787	2.402505
321	0.042054	2.641969	349	0.031460	2.394354
322	0.041618	2.632377	350	0.031138	2.386250
323	0.041187	2.622845	351	0.030818	2.378193
324	0.040760	2.613373	352	0.030502	2.370182
325	0.040338	2.603959	353	0.030190	2.362217
326	0.039921	2.594603	354	0.029880	2.354297
327	0.039508	2.585306	355	0.029574	2.346423
328	0.039100	2.576065	356	0.029271	2.338593
329	0.038695	2.566881	357	0.028972	2.330807
330	0.038296	2.557753	358	0.028675	2.323066
331	0.037900	2.548681	359	0.028382	2.315368
332	0.037509	2.539664	360	0.028091	2.307713
333	0.037122	2.530702	361	0.027804	2.300101
334	0.036739	2.521794	362	0.027520	2.292532
335	0.036360	2.512939	363	0.027238	2.285005
336	0.035985	2.504138	364	0.026960	2.277519
337	0.035614	2.495389	365	0.026685	2.270075
338	0.035248	2.486693	366	0.026412	2.262673
339	0.034885	2.478049	367	0.026142	2.255311
340	0.034525	2.469455	368	0.025876	2.247989
341	0.034170	2.460913	369	0.025612	2.240708
342	0.033819	2.452421	370	0.025350	2.233466
343	0.033471	2.443979	371	0.025092	2.226264
344	0.033127	2.435587	372	0.024836	2.219101
345	0.032786	2.427244	373	0.024583	2.211977
346	0.032450	2.418949	374	0.024332	2.204891

APPENDIX—contd.

n	$\frac{1}{e^{n/100} - 1}$	$\frac{1}{e^{n/1,000} - 1}$	n	$\frac{1}{e^{n/100} - 1}$	$\frac{1}{e^{n/1,000} - 1}$
375	0.024084	2.197844	403	0.018096	2.014882
376	0.023839	2.190834	404	0.017913	2.008823
377	0.023596	2.183862	405	0.017731	2.002794
378	0.023356	2.176928	406	0.017552	1.996795
379	0.023118	2.170030	407	0.017374	1.990826
380	0.022883	2.163170	408	0.017198	1.984886
381	0.022650	2.156345	409	0.017024	1.978976
382	0.022419	2.149557	410	0.016852	1.973096
383	0.022191	2.142805	411	0.016681	1.967244
384	0.021966	2.136088	412	0.016513	1.961421
385	0.021742	2.129407	413	0.016346	1.955627
386	0.021521	2.122761	414	0.016180	1.949861
387	0.021303	2.116149	415	0.016017	1.944123
388	0.021086	2.109572	416	0.015855	1.938413
389	0.020872	2.103029	417	0.015695	1.932731
390	0.020660	2.096520	418	0.015536	1.927077
391	0.020450	2.090045	419	0.015379	1.921450
392	0.020243	2.083604	420	0.015224	1.915850
393	0.020037	2.077195	421	0.015070	1.910277
394	0.019834	2.070820	422	0.014918	1.904731
395	0.019633	2.064477	423	0.014767	1.899212
396	0.019434	2.058167	424	0.014618	1.893718
397	0.019236	2.051888	425	0.014471	1.888252
398	0.019041	2.045642	426	0.014325	1.882811
399	0.018848	2.039428	427	0.014180	1.877396
400	0.018657	2.033245	428	0.014037	1.872007
401	0.018468	2.027093	429	0.013895	1.866643
402	0.018281	2.020972	430	0.013755	1.861305

APPENDIX—contd.

n	$\frac{1}{e^{n/100} - 1}$	$\frac{1}{e^{n/1,000} - 1}$	n	$\frac{1}{e^{n/100} - 1}$	$\frac{1}{e^{n/1,000} - 1}$
431	0·013616	1·855992	459	0·010257	1·716766
432	0·013479	1·850703	460	0·010154	1·712112
433	0·013343	1·845440	461	0·010052	1·707479
434	0·013209	1·840201	462	0·009951	1·702866
435	0·013076	1·834987	463	0·009851	1·698273
436	0·012944	1·829797	464	0·009752	1·693701
437	0·012813	1·824631	465	0·009654	1·689149
438	0·012684	1·819489	466	0·009557	1·684616
439	0·012556	1·814371	467	0·009461	1·680104
440	0·012430	1·809276	468	0·009366	1·675611
441	0·012305	1·804205	469	0·009272	1·671137
442	0·012181	1·799157	470	0·009179	1·666683
443	0·012058	1·794133	471	0·009087	1·662248
444	0·011937	1·789131	472	0·008995	1·657832
445	0·011817	1·784153	473	0·008905	1·653435
446	0·011698	1·779196	474	0·008816	1·649058
447	0·011580	1·774263	475	0·008727	1·644698
448	0·011463	1·769352	476	0·008640	1·640358
449	0·011348	1·764463	477	0·008553	1·636036
450	0·011234	1·759596	478	0·008467	1·631733
451	0·011121	1·754751	479	0·008382	1·627448
452	0·011009	1·749928	480	0·008298	1·623181
453	0·010898	1·745127	481	0·008215	1·618932
454	0·010789	1·740347	482	0·008132	1·614701
455	0·010680	1·735589	483	0·008051	1·610489
456	0·010573	1·730851	484	0·007970	1·606292
457	0·010466	1·726135	485	0·007890	1·602115
458	0·010361	1·721440	486	0·007811	1·597955

APPENDIX—contd.

n	$\frac{1}{e^{n/100} - 1}$	$\frac{1}{e^{n/1,000} - 1}$	n	$\frac{1}{e^{n/100} - 1}$	$\frac{1}{e^{n/1,000} - 1}$
487	0.007733	1.593812	515	0.005833	1.484476
488	0.007655	1.589686	516	0.005775	1.480795
489	0.007578	1.585578	517	0.005717	1.477129
490	0.007502	1.581487	518	0.005660	1.473477
491	0.007427	1.577413	519	0.005603	1.469839
492	0.007353	1.573356	520	0.005547	1.466216
493	0.007279	1.569315	521	0.005492	1.462607
494	0.007206	1.565292	522	0.005437	1.459013
495	0.007134	1.561285	523	0.005382	1.455432
496	0.007062	1.557294	524	0.005328	1.451865
497	0.006992	1.553320	525	0.005275	1.448312
498	0.006922	1.549362	526	0.005222	1.444773
499	0.006852	1.545420	527	0.005170	1.441248
500	0.006784	1.541494	528	0.005118	1.437736
501	0.006716	1.537584	529	0.005067	1.434238
502	0.006648	1.533691	530	0.005017	1.430754
503	0.006582	1.529813	531	0.004966	1.427283
504	0.006516	1.525950	532	0.004917	1.423825
505	0.006451	1.522104	533	0.004868	1.420380
506	0.006386	1.518272	534	0.004819	1.416940
507	0.006322	1.514457	535	0.004771	1.413531
508	0.006259	1.510656	536	0.004723	1.410126
509	0.006196	1.506871	537	0.004676	1.406734
510	0.006134	1.503101	538	0.004629	1.403355
511	0.006073	1.499346	539	0.004583	1.399988
512	0.006012	1.495606	540	0.004537	1.396635
513	0.005952	1.491881	541	0.004492	1.393294
514	0.005892	1.488171	542	0.004447	1.389966

APPENDIX—contd.

n	$\frac{1}{e^{n/100} - 1}$	$\frac{1}{e^{n/1,000} - 1}$	n	$\frac{1}{e^{n/100} - 1}$	$\frac{1}{e^{n/1,000} - 1}$
543	0.004402	1.386650	571	0.003324	1.298640
544	0.004358	1.383347	572	0.003291	1.295661
545	0.004315	1.380056	573	0.003258	1.292691
546	0.004272	1.376777	574	0.003225	1.289733
547	0.004229	1.373511	575	0.003193	1.286785
548	0.004187	1.370257	576	0.003161	1.283848
549	0.004145	1.367015	577	0.003130	1.280921
550	0.004104	1.363786	578	0.003098	1.278004
551	0.004063	1.360568	579	0.003067	1.275098
552	0.004022	1.357362	580	0.003037	1.272202
553	0.003982	1.354168	581	0.003006	1.269317
554	0.003942	1.350986	582	0.002976	1.266441
555	0.003903	1.347816	583	0.002947	1.263576
556	0.003864	1.344658	584	0.002917	1.260721
557	0.003825	1.341511	585	0.002888	1.257876
558	0.003787	1.338375	586	0.002859	1.255041
559	0.003749	1.335251	587	0.002831	1.252216
560	0.003712	1.332139	588	0.002803	1.249400
561	0.003675	1.329038	589	0.002775	1.246595
562	0.003638	1.325948	590	0.002747	1.243799
563	0.003601	1.322870	591	0.002720	1.241013
564	0.003566	1.319802	592	0.002692	1.238237
565	0.003530	1.316746	593	0.002666	1.235470
566	0.003495	1.313701	594	0.002639	1.232713
567	0.003460	1.310667	595	0.002613	1.229966
568	0.003425	1.307644	596	0.002587	1.227227
569	0.003391	1.304632	597	0.002561	1.224499
570	0.003357	1.301631	598	0.002535	1.221780

APPENDIX—contd.

n	$\frac{1}{e^{n/100} - 1}$	$\frac{1}{e^{n/1,000} - 1}$	n	$\frac{1}{e^{n/100} - 1}$	$\frac{1}{e^{n/1,000} - 1}$
599	0.002510	1.219070	735	0.000643	0.921250
600	0.002485	1.216369	740	0.000612	0.912462
605	0.002363	1.203004	745	0.000582	0.903798
610	0.002248	1.189865	750	0.000553	0.895255
615	0.002138	1.176946	755	0.000526	0.886830
620	0.002034	1.164242	760	0.000501	0.878521
625	0.001934	1.151747	765	0.000476	0.870326
630	0.001840	1.139458	770	0.000453	0.862243
635	0.001750	1.127368	775	0.000431	0.854269
640	0.001664	1.115473	780	0.000410	0.846402
645	0.001583	1.103769	785	0.000390	0.838639
650	0.001506	1.092251	790	0.000371	0.830981
655	0.001432	1.080915	795	0.000353	0.823424
660	0.001362	1.069756	800	0.000336	0.815966
665	0.001296	1.058772	805	0.000319	0.808606
670	0.001232	1.047957	810	0.000304	0.801341
675	0.001172	1.037309	815	0.000289	0.794170
680	0.001115	1.026823	820	0.000275	0.787092
685	0.001061	1.016496	825	0.000261	0.780104
690	0.001009	1.006324	830	0.000249	0.773205
695	0.000960	0.996305	835	0.000236	0.766393
700	0.000913	0.986434	840	0.000225	0.759667
705	0.000868	0.976709	845	0.000214	0.753025
710	0.000826	0.967126	850	0.000204	0.746465
715	0.000785	0.957683	855	0.000194	0.739987
720	0.000747	0.948377	860	0.000184	0.733589
725	0.000711	0.939204	865	0.000175	0.727270
730	0.000676	0.930163	870	0.000167	0.721027

APPENDIX—concl'd.

n	$\frac{1}{e^{n/100} - 1}$	$\frac{1}{e^{n/1,000} - 1}$	n	$\frac{1}{e^{n/100} - 1}$	$\frac{1}{e^{n/1,000} - 1}$
875	0.000158	0.714860	940	0.000083	0.641033
880	0.000151	0.708768	945	0.000079	0.635803
885	0.000143	0.702748	950	0.000075	0.630632
890	0.000136	0.696801	955	0.000071	0.625520
895	0.000130	0.690925	960	0.000068	0.620464
900	0.000123	0.685118	965	0.000064	0.615465
905	0.000117	0.679379	970	0.000061	0.610521
910	0.000112	0.673708	975	0.000058	0.605632
915	0.000106	0.668103	980	0.000055	0.600797
920	0.000101	0.662563	985	0.000053	0.596014
925	0.000096	0.657087	990	0.000050	0.591284
930	0.000091	0.651674	995	0.000048	0.586605
935	0.000087	0.646323	1,000	0.000045	0.581977

A PAPAIN-CASEIN CULTURE MEDIUM FOR THE PREPARATION OF BACTERIOPHAGE, AND FOR GENERAL LABORATORY USE.

BY

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INTRODUCTION.

SOME experiments (unpublished) conducted by us showed that, so far as could be judged by plaque counts, cholera bacteriophage not only grew more profusely but the various types survived longer in 1 per cent peptone (Witte's) medium than in papain-mutton broth (Martin, 1927; Morison and Vardon, 1929) which was the routine medium used in this Institute for the preparation of therapeutic cholera-dysentery bacteriophage. It was thought possible that this was because the nitrogenous content of papain-mutton broth is represented chiefly by free amino-acids owing to the complete digestion of the mutton in the process of preparation.

It would have been a very simple matter to have adopted a 1 per cent Witte's peptone medium for the preparation of therapeutic bacteriophage, but there are two great objections to this, namely, the relatively high cost of commercial peptones and, secondly, the fact that most peptones are derived from sources such as ox-blood fibrin, which would be objectionable to the majority of the people of India.

Our object, therefore, was to try and obtain a medium in which the nitrogen content would be represented mainly by proteose and peptone and which was derived from a source non-objectionable to the masses in India, and also which would be no more expensive than papain-mutton broth.

At the suggestion of Lieut.-Colonel L. A. P. Anderson, I.M.S., Director of this Institute, work on the digestion of casein by papain was taken up by us. It would seem that, if this proved successful in other respects our problem would be solved since neither casein nor papain, which is the enzyme present in the fruit *Carica papaya*, could be in any way objectionable even to the most orthodox.

Casein, the chief protein of milk, contains a large number of amino-acids including tryptophane, which is particularly necessary for the growth of certain bacteria and the use of casein as the nitrogen basis of bacterial culture media is, of course, not new. For instance, Cole and Onslow (1916) prepared a culture medium by the digestion of casein with trypsin; this medium however was found unsuitable for most pathogenic bacteria (Fildes and McIntosh, 1931).

Norris (1920) in India also prepared a trypsinized casein medium and found that the growth of bacteria was as good in this medium as in trypsinized mutton broth, whilst the cost of production was less. She could not, however, obtain a perfectly clear broth and the medium was consequently only really suitable for the preparation of agar for large scale vaccine manufacture. Actually this medium was used successfully for this purpose for many years at the Central Research Institute, Kasauli.

Kristensen *et al.* (1925) prepared a trypsinized casein broth and observed that it was suitable for the growth of pathogenic bacteria of the typhoid-paratyphoid group, an excellent medium for the indol test and very cheap; their process of preparation was a lengthy one and took more than a week for each brew.

DIGESTION OF CASEIN WITH PAPAIN.

Materials.—Preliminary experiments were conducted with a sample of commercial casein (Merck's). Afterwards crude casein was obtained from a firm in Poona, India. On estimation of the total nitrogen the latter sample was found to contain 76.25 per cent protein.

The papain, in the form of the dried juice, was obtained from Ceylon. When shaken with water it gave the usual milky appearance; the reaction was acid, pH 5.2, and the Biuret test was positive. The filtered hot-water extract when analysed showed that papain contains 8.8 per cent soluble nitrogen. Assuming this nitrogen to be derived from protein alone, the ferment therefore contained 55 per cent soluble protein.

In the preliminary experiments and subsequent work the degree of digestion of the casein was measured by Sorensen's formol titration method using Cole and Onslow's comparator. After digestion 20 c.c. of the digest were taken and 5 c.c. of neutral formalin (70 c.c. formaldehyde 40 volumes per cent made faintly pink to phenolphthalein and diluted to 100 c.c.) were added and this was titrated with N/10 NaOH (caustic soda) using phenolphthalein as the indicator.

Summary of preliminary experiments.—Preliminary observation had shown that when digestion was carried out for not more than six hours at different temperatures and reactions, free tryptophane was never detected in the digest and that up to this period papain digested casein only to the proteose and peptone stage. At 70°C. to 80°C. 1.5 g. papain digested one litre of 2.5 per cent

casein solution in approximately two hours. At 85°C. the enzyme was quickly inactivated. In our subsequent experiments 70°C. was adopted as the optimum temperature for digestion.

When the digestion of casein was carried out at different hydrogen-ion concentrations, it was found that digestion was most rapid at pH 5.0. It may be noted that the iso-electric point of the particular sample of casein was found to be pH 4.8. To obtain a clear broth for a culture medium of pH 7.8, digestion at pH 5.0, however, had the disadvantage that more adjustments of the reaction were necessary. Digestion at pH 7.8 to pH 8.0, on the other hand, favoured the deposition of inorganic phosphates which are disintegrated during the process and necessitated only a little subsequent adjustment of the pH, consequently digestion at pH 5.0 was discontinued and pH 7.8 was adopted as the initial reaction for the digestion of casein by papain, for the purpose of preparing a medium for bacteriological experimental work.

DRIED PAPAYA JUICE ALONE, AS A NUTRIENT MEDIUM FOR BACTERIA.

It has already been mentioned that papain (the dried juice) has a high protein content, it was also found to give a positive test for reduced glutathione, a tripeptide containing the amino-acids glycine, cysteine, and glutamic acid which favour the growth of certain bacteria; this suggested that in the preparation of papain-casein medium, in addition to the digestive property of the papain, a part of the nutrients contained in the papain might also be available to supplement those obtained from the casein. To clear up this point the following experiment was undertaken:—

Powdered papain mixed in distilled water was autoclaved for 45 minutes at 15 lb. pressure to obtain complete solution and to destroy the ferment activity. Sodium chloride to 0.5 per cent was then added and the pH of the fluid was adjusted to 7.8. It was then steamed for 30 minutes, cooled, and filtered. Sterilized filtrates containing different concentrations of papain were used for growing strains of *V. cholera* and it was found that the organisms definitely multiplied in a 0.375 per cent solution of papain.

In preparing the digest for the culture medium, therefore, 7.5 g. (1.5×5) of papain per litre of 2.5 per cent casein solution were used so that when the final dilution of the digest with an equal volume of water is made the amount of papain in the finished medium would be 0.375 per cent. Moreover, although it was previously found that 1.5 g. of papain would digest one litre of 2.5 per cent casein, it was considered that, by using five times the quantity of the ferment which was actually required, there would be available a sufficient margin to compensate for any possible variation in the quality of the papain.

PREPARATION OF PAPAIN-CASEIN CULTURE MEDIUM.

One litre of a 0.8 per cent solution of anhydrous sodium carbonate in tap water is placed in a 4-litre flask and the temperature of the solution is raised to 70°C. in a water-bath. To this hot alkaline solution is added 100 g. of crude casein (made

from skimmed milk) and the heating is continued for about 20 minutes or until the casein is almost completely dissolved. The flask is then shaken vigorously and the volume is made up to 4,000 c.c. with tap water. At this stage the casein mixture should show a pH of 8.0 to 8.5*. The flask is then replaced in the bath and when the temperature of the fluid in the flask reaches 70°C., 30 g. of papain are added and well mixed. On the addition of the papain the pH of the fluid drops to about 7.8. The digestion of the casein is continued in the bath for two hours, the temperature being maintained at 70°C.

At the end of two hours, 20 c.c. of the digest are removed and cooled under the tap. This is titrated with N/10 caustic soda, after adding 5 c.c. neutral formalin and 25 drops of 0.5 per cent phenolphthalein solution. When digestion is complete to the desired stage about 9 c.c. of the alkali are required to give a distinct pink coloration. Based on the above method of preparation the table shows the progress of digestion at various times:—

TABLE I.
Course of digestion of casein by papain.

Time in minutes.	Digestion temperature, °C.	Reaction of the digest, pH.	c.c. of N/10 NaOH required to neutralize.	Stage of digestion as shown by Biuret test.
Initial	70	7.8	3	Violet (undigested protein).
15	70	7.3	8	Rose-red (peptones).
30	69	7.3	8.8	Rose-red colour deepens.
60	69	7.2	9.3	..
90	70	7.2	9.6	..
120	71	7.2	9.65	..

Having determined that digestion is complete the flask is removed from the bath and the digest is filtered through a piece of thick cloth or canvas. The

* Since the acidity of commercial samples of casein varies somewhat, the initial amount of Na_2CO_3 necessary to give a final pH of 8.0 to 8.5 may, of course, also vary slightly.

For large scale preparation enamelled milk cans may with advantage be used for the digestion process instead of glass flasks.

filtrate is still turbid, the turbidity being chiefly due to the presence of inorganic phosphates.

The reaction of the filtrate (pH 7.0 to pH 7.2) is then adjusted to pH 7.6, using phenol red as indicator; about 2 c.c. N/10 NaOH per litre are required.

The medium is then either steamed for 30 minutes, or autoclaved, to inactivate the ferment and to deposit the phosphates. The pH, after the above heating, is usually between 7.8 and 8.0.

On cooling, when the supernatant is completely clear, the contents are filtered through filter-paper without disturbing the deposit which tends to choke the pores of the filter-paper and makes the filtration slow; later the deposit is placed on the paper.

The clear filtrate is diluted* with an equal volume of tap water and 5 g. sodium chloride per litre are added. The pH of the medium remains constant at pH 7.8 to 8.0.

The medium can be tubed or placed in flasks and sterilized. Before this sterilization, formol titration of 20 c.c. of the medium requires approximately 3.9 c.c. to 4 c.c. N/10 NaOH. The drop from 9.65 c.c. to 4 c.c. in the amount of caustic soda required is due to the dilution of the original papain-casein digest and the subsequent addition of alkali and heating when possibly some neutral salts are formed.

Papain-casein broth can be used for the preparation of nutrient agar, blood agar, and Loeffler's serum medium.

Nutrient agar.—The agar fibre, 2.5 per cent, is dissolved in the papain-casein broth, just after the dilution and the addition of sodium chloride. The filtration through lint and Chardin filter-paper should be performed at a comparatively low temperature, about 60°C., otherwise on cooling a thin white deposit of inorganic salts (which, however, do not interfere with the growth) may be formed at the bottom of the agar slopes. 'Metasil' filtration which is carried out at this Institute gives a clear nutrient agar. The pH of the prepared agar is found to be the same as that of the papain-casein broth used.

ANALYSIS OF THE PAPAIN-CASEIN MEDIUM.

Papain-casein broth, Witte's peptone 1 per cent solution and papain-mutton broth, of pH 7.8, sterilized and ready for use were next analysed. In the case of papain-mutton broth, only the total nitrogen and the amino-acid nitrogen were estimated. All the analyses were carried out under parallel conditions. The method adopted for the determination of the quantities of proteose and peptone was essentially that of Wastneys and Borsook (1924).

* This was done so that the composition of the finished medium might be approximately similar to that of one per cent Witte's peptone solution. If, however, it is found in the course of routine laboratory work that the growth obtained is not as good as one is accustomed to on other media, the amount of tap water added might be reduced.

TABLE II.

Analysis of peptone solution, papain-casein broth, and papain-mutton broth.
Mg. per 100 c.c.

Constituent.	Witte's peptone 1 per cent solution.	Papain-casein broth. 1·25 per cent casein, 0·375 per cent papain.	Papain-mutton broth. 8·5 per cent mutton, 0·5 per cent papain.
Total nitrogen	155 mg.	185 mg.	245 mg.
Proteose nitrogen	60 „	85 „	..
Peptone nitrogen	55 „	59 „	..
Amino-acid nitrogen	16 „	31 „	82 mg.
Other nitrogen combinations ..	24 „	10 „	..
Chlorides (as NaCl, including 0·5 per cent added).	0·585 g.	0·58 g.	0·6 g.
Reducing substance in term of glucose.	0	0·008 g.	..
Calcium	8 mg.	10·5 mg.	..

It will be seen from Table II that papain-casein medium prepared as described above is rich in proteose and peptone and compares favourably in respect of peptone nitrogen with 1 per cent peptone (Witte's) solution.

COST OF PRODUCTION OF PAPAIN-CASEIN MEDIUM.

In comparison with papain-mutton medium, the preparation of papain-casein broth saves time and labour in that the digestion is complete within two hours, whereas for the papain-mutton broth, besides the extra time and labour spent in obtaining a bone-and-fat-free mince, the digestion has to be carried on for six hours on the first day and for at least two hours more on the second day and the pH of the digest has to be adjusted hourly; this is not necessary in the case of papain-casein medium.

The following is a comparative statement of the cost of raw materials required per brew of 72 litres of finished broth, which is the quantity prepared at one time for routine use in this Institute for the manufacture of bacteriophage, vaccine, etc.

TABLE III.

Comparative cost of media.

Medium.	Quantities of raw materials used.	Cost per brew of 72 litres finished broth.
		Rs. d. p.
Witte's peptone (1 per cent)	1½ lb.	39 0 0
Papain-mutton broth ..	<div> Mutton, 24 lb. Papain, 14 oz. </div>	13 8 0
Papain-casein broth ..	<div> Casein, 2 lb. Papain, 9 oz. </div>	4 10 0

Annually between 100 to 125 such brews of medium are required.

THE SUITABILITY OF THE MEDIUM FOR BACTERIOLOGICAL WORK.

Having obtained a medium rich in proteose and peptone, which was cheap and non-objectionable, the other factors which had to be determined were :—

- Suitability of the medium for growth of ordinary bacteria,
- Suitability for growth of delicate bacteria, and
- Suitability for growth of bacteriophage.

These three factors were then investigated.

(a) Suitability of the medium for growth of ordinary bacteria.

B. coli, *V. cholerae*. *B. pseudo-carolinus*, *B. paratyphosus* A and B, and *B. typhosus* were all grown on papain-mutton and papain-casein agar.

The growth of all the above on papain-casein agar slopes was good and the morphology was typical.

Three strains of cholera vibrios were then sown in three Roux flasks each containing 200 c.c. of papain-casein agar. Each flask was sown with one of the strains and the same amount of broth culture was added.

Three similar flasks of papain-mutton agar were sown at the same time as controls. These flasks were incubated for 48 hours, after which 15 c.c. of normal saline were added to each flask and the growth was thoroughly washed off and collected in separate test tubes.

The yield was estimated both by the opacity test and by taking the dry weight of organisms washed as free as possible from extraneous matter.

TABLE IV.

Cholera culture. Yield per flask.

Cholera vibrio strain.	PAPAIN-CASEIN AGAR.				PAPAIN-MUTTON AGAR.			
	Total washing, c.c.	Strength in millions per c.c. (by opacity).	Dry weight, mg. per c.c.	Total harvest in g.	Total washing, c.c.	Strength in millions per c.c. (by opacity).	Dry weight, mg. per c.c.	Total harvest in g.
U. P. I. ..	27	38,241	7.77	0.21	29	45,892	9.31	0.27
Manipur 6 ..	27	45,892	9.31	0.25	27	45,892	9.31	0.25
Manipur 57 .	30	45,892	9.31	0.28	28	45,892	9.31	0.26
Average yield : casein-agar flasks = 0.246 g.					Average yield : mutton-agar flasks = 0.26 g.			

Subsequent to the above experiment, two brews of cholera vaccine were prepared by sowing 103 Roux flasks in each brew with four strains of *V. cholerae*. The organisms were first sown in four flasks of papain-casein broth and these broth flasks were used as 'seed' for the Roux flasks, each of which contained 200 c.c. of 2.5 per cent papain-casein agar.

The harvest of these two brews yielded an average per Roux flask of 181.5 c.c. of vaccine of a strength of 8,000 millions per c.c. The average yield per flask of ten of the most recent brews of cholera vaccine grown on 2.5 per cent papain-mutton agar was 173.7 c.c.

For culturing ordinary aerobic pathogenic bacteria and for cholera vaccine preparation casein-papain medium was thus proved to be very satisfactory.

Further experiments were carried out to determine the suitability of papain-casein broth as the basic medium for fermentation, indol and cholera-red reactions, instead of the much more costly peptone water ordinarily used.

The tests were carried out with the same organisms as before. The sugars gave correct readings after 24 hours and four days' incubation, but many more tubes with this medium showed fluorescence than with the control tubes prepared from Witte's peptone and this made the reading of results very difficult. The indicator used was neutral red, but Kristensen *et al.* (*loc. cit.*) when working with trypsinized casein found that Brom-thymol-blue was the best indicator for fermentation tests in fluid media. It is possible that by the use of Brom-thymol-blue this difficulty would be overcome.

The indol reaction was given by *B. coli* and *V. cholerae* but not by *B. typhosus* or *B. paratyphosus* A or B. The cholera-red reaction with a true cholera strain was negative, but this very often occurs with Witte's peptone water. When 0.01 per cent sodium nitrate was added to the medium a well-marked cholera-red reaction was obtained. At present 0.01 per cent sodium nitrate is being added to the Witte's peptone water tubes which are used for the cholera-red reaction.

(b) Suitability for growth of delicate bacteria.

Two streptococcus strains, one pneumococcus and a gonococcus culture, were sub-cultured on papain-mutton and on papain-casein blood agar and incubated for 48 hours.

There was no appreciable difference in the amount of growth of the streptococcus and pneumococcus cultures on the two media.

The morphology of both the streptococcus and the pneumococcus which were grown on papain-casein blood agar was typical. The gonococcus culture showed a more profuse growth on papain-casein blood agar, and in Gram-stained smears the morphology was typical and indeed the characteristic appearance of the organism was better marked than in smears from a culture on papain-mutton agar which showed many pleomorphic forms.

A test was also carried out to ascertain the length of time a delicate organism would live on papain-casein medium without sub-culturing.

Three tubes each of papain-mutton blood agar and papain-casein blood agar were sub-cultured from a gonococcus culture. After 48 hours' incubation, one tube of each was placed in the incubator at 37°C., one on the laboratory bench at room temperature (about 22°C.), and one in the frigidaire. After every 24 hours sub-cultures were made from these cultures on to papain-mutton blood agar and papain-casein blood agar slopes and the inoculated slopes after being incubated at 37°C. for 48 hours were examined for growth. The results are noted below.

A hæmolytic streptococcus was also tested, keeping it all the time in the incubator and it was found to be alive after six days on both media. These experiments show that papain-casein medium is suitable for the growth of these more delicate organisms.

TABLE V.

Date.	INCUBATOR.		ROOM.		FRIGIDAIRE.	
	Mutton.	Casein.	Mutton.	Casein.	Mutton.	Casein.
10-10-1936 ..	+	+	±	±	—	—
11-10-1936 ..	+	+	±	±	—	—
12-10-1936 ..	+	+	—	Few scattered colonies.	—	—
13-10-1936 ..	+	+	—	Do.	—	—
14-10-1936 ..	+	+	—	—	—	—

Note.— + indicates that a good growth was obtained.

± Scanty growth.

— No growth.

Finally Loeffler's medium was also prepared with papain-casein broth basis in order to test its suitability for growing *C. diphtheriæ*.

This organism grew profusely on the medium and stained smears showed that its morphology was as typical as in smears made from cultures on the ordinary Loeffler's medium used in this Institute.

(c) *Suitability for the growth of bacteriophage.*

Two brews of cholera-dysentery bacteriophage for therapeutic use were prepared from papain-casein broth. The lysis in the flasks after 24 hours' incubation was quite satisfactory in the case of both the dysentery and cholera culture flasks.

The results of the analyses of these to brews as compared with the three previous brews prepared in papain-mutton broth were as follows:—

TABLE VI.

Brew number.	Titre against cholera.	Titre against dysentery.	Number of cholera-phage types present in 10^{-1} dilution.	Number of cholera-phage types present in 10^{-10} dilution.
486 (Mutton)	$10^{-8}+++$ $10^{-10}++$, $10^{-12}+$	$10^{-12}+++$	8	8
487 (Mutton) ..	$10^{-12}+++$	$10^{-12}+++$	7	6
488 (Mutton)	$10^{-5}+++$ 10^{-6} to $10^{-10}++$ $10^{-12}+$	$10^{-12}+++$	7	4
489 (Casein) ..	$10^{-12}+++$	$10^{-12}+++$	9	5
490 (Casein) ..	$10^{-12}+++$	$10^{-12}+++$	8	7

Note.— $+++$
 $++$
 $+$ } indicate degrees of lysis. ($+++$ = complete lysis.)

Having shown that cholera-dysentery bacteriophage could be prepared in the papain-casein medium, a final test was carried out to ascertain whether the cholera-phage types remained viable for as long a period in this medium as in Witte's peptone water 1 per cent.

The casein for the test medium was prepared from two samples of commercial casein, one, Merck's and the other of local Indian manufacture.

The three media, papain-casein broth (Merck's), papain-casein broth (Indian), and Witte's peptone water, were adjusted to pH 7.8 and each was sown with the same dose of the same phage seed and cholera culture. After sowing, the flasks were incubated for 24 hours at 37°C . and the lysed products were filtered separately through L3 candles.

Dilution tests of each filtrate were then put up and each dilution tube was filtered separately and tested for the cholera-phage types present. The filtrates were then ampouled and the ampoules were stored in the incubator at 37°C . and tested at intervals of about a month, during the next six months.*

Tables VII and VIII show the results of these tests.

* From previous experiments carried out by us it was found that cholera-phage types died out much sooner at 37°C . than at room temperature or in the frigidaire. The ampoules were, therefore, stored at 37°C . to get rapid conclusive results.

TABLE VII.

Date of test.	CASEIN (MERCK'S).										WITTE'S PEPTONE.										Total number of types present.
	Cholera-phage types present.										Cholera-phage types present.										
A	B	C	D	E	F	G	H	J	K	A	B	C	D	E	F	G	H	J	K		
31-8-1936	++	±	++	-	++	++	±	++	++	++	+	++	-	++	++	±	++	++	++	++	9
	12	1	12	..	12	12	1	12	2	12	12	2	12	..	12	12	1	12	2	10	
30-9-1936 ..	++	±	++	-	++	++	-	++	±	++	±	++	-	++	++	-	++	+	++	8	
5-11-1936 ..	++	-	±	-	++	++	-	++	±	+	±	-	-	++	++	-	++	-	-	4	
4-12-1936 ..	++	-	-	-	++	+	-	++	-	-	-	-	-	++	+	-	++	-	-	4	
19-1-1937 ..	+	-	-	-	++	+	-	++	-	-	-	-	-	++	+	-	++	-	-	4	
4-2-1937 ..	+	-	-	-	++	+	-	++	-	-	-	-	-	++	+	-	++	-	-	4	

Note.—(1) ++ denotes a completely lysed area.
+ " confluent and semi-confluent plaques.
± " discrete plaques (few).
(2) The figures below the + signs in the first line denote the decimal dilutions in which each type of phage was present in the original filtrate. Thus the figure 12 means that this particular type was present in 10⁻¹² dilution.

TABLE VIII.

Date of test.	CASEIN (INDIAN).											WITTE'S PERTONE.											Total number of types present.
	Cholera-phage types present.											Cholera-phage types present.											
	A	B	C	D	E	F	G	H	J	K	A	B	C	D	E	F	G	H	J	K			
4-10-1936	++	+	++	±	++	++	±	++	+	++	10	++	±	++	±	++	-	++	±	++	9		
	12	1	7	1	12	12	1	12	1	12		12	1	7	1	12	12	..	12	1		6	
7-11-1936 ..	++	-	++	-	++	++	±	++	-	++	7	++	-	-	++	++	-	++	-	++	5		
4-12-1936 ..	++	-	-	-	++	++	±	++	-	+	6	++	-	-	++	++	-	++	-	-	4		
19-1-1937 ..	++	-	-	-	++	++	±	++	-	-	5	++	-	-	++	++	-	++	-	-	4		
4-12-1937 ..	++	-	-	-	++	++	-	++	-	-	4	++	-	-	++	++	-	++	-	-	4		
18-3-1937 ..	+	-	-	-	++	++	-	++	-	-	4	++	-	-	++	±	-	++	-	-	4		

Note.—(1) ++ denotes a completely lysed area.

++ " confluent and semi-confluent plaques.

± " discrete plaques (few).

(2) The figures below the + signs in the first line denote the decimal dilutions in which each type of phage was present in the original filtrate. Thus the figure 12 means that this particular type was present in 10^{-12} dilution.

From the above tables it will be seen that both samples of casein medium gave somewhat better results than Witte's peptone water, in that during the first three months of storage more types died out in the peptone medium than in the papain-casein digest medium.

SUMMARY.

The test carried out on the papain-casein digest medium which we have prepared have shown that this is a satisfactory culture medium. It is the cheapest medium so far produced in this laboratory and it is entirely non-objectionable. It is suitable for the growth of ordinary and delicate organisms, and it has also proved to be a very satisfactory medium for the preparation of therapeutic bacteriophage and cholera vaccine. Cholera phage types survived longer in this medium than in Witte's peptone water and previous experiments had shown that in Witte's peptone water cholera phage types survived longer than in papain-mutton broth.

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ON WASSERMANN REACTION.

Part III.*

THE POSITIVE SERUM.

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A KNOWN fully positive serum left over from the previous day's test is used as a control in a day's work. It has been recommended that a weakly positive serum should also be used as a control. Such a serum is not found frequently. The recommendation, therefore, is usually ignored. For testing the suitability of a new stock of antigen a known fully positive serum is again required. Some cautious workers test a new antigen with several positive sera left over from the previous day's work.

The senior writer commenced a study of the various antigens used in Wassermann reaction several years ago. One point concerning the positive control emerged during the course of the study. The positive serum varies in its power to fix complement within limits so wide that the ordinary positive control, of the usual 1 in 5 dilution of a known serum, is a very poor measure for a quantitative reading of the reaction of the unknown sera in a day's work and is utterly useless in comparing stocks of new antigens; the antigens selected by such a standard may not be comparable even for qualitative work. In view of the importance of the

* For Part II see *Indian Journal of Medical Research*, 17, No. 4, 1930, p. 1161.

point and the possibility that it may be lost in the mass of observations on Wassermann antigen this independent communication has been decided upon.

THE DEGREE OF POSITIVENESS OF A SERUM. AS DEFINED BY (BRITISH)
MEDICAL RESEARCH COMMITTEE (1918, NOW COUNCIL).

The following notation is suggested in method number four:—

(a) Complete inhibition with 3 M. H. D., and 5 M. H. D. of complement
= + +.

(b) Complete inhibition with 3 M. H. D., partial inhibition with 5 M. H. D.
= + ±.

(c) Complete inhibition with 3 M. H. D., complete lysis with 5 M. H. D. = +.

(d) Slight lysis with 3 M. H. D. = ±.

(e) Considerable lysis with 3 M. H. D. = ±.

The writers after prolonging the final incubation to 30 minutes record the reactions in accordance with the notation but report:—

+ + as positive (or fully positive).

+ ± and + as weakly positive.

± and ± as doubtful.

The writers also record ? — which is reported as negative. Between ± and ? — there is not much difference. Both these reactions are judged by turbidity on the first day. Next day, after the tubes have been left in the cold overnight, there should be a definite deposit of red blood cells in them and a distinct difference of colour between them and the negative control. If not, they are recorded as ? — and reported as negative.

Wyler (1929) in his modification of method number one ignores a trace of lysis in one of the tubes and reports a + in its place. His reports are:—

+ + = strongly positive.

+ = positive.

± = positive in known cases of syphilis and doubtful in an unknown case.

Wyler also recognizes the fact that there may be some degree of inhibition of lysis in both tubes. He reads such reactions + or ± depending upon the inhibition.

The writers also read T T as traces of lysis, both with 3 M. H. D. and 5 M. H. D. These reactions, along with those yielding some degree of inhibition of lysis in both tubes, the senior writer had described (Greval, 1930), before Wyler's publication became available in India, as 'irregular reactions' and classed them with doubtful reactions on clinical grounds. Since then he has observed similar reactions in titrating, with their appropriate antigens by complement-fixation, weak immune sera produced in animals for therapeutical purposes (Greval, 1933a and b). He has also observed them in tropical diseases other than yaws, appearing and disappearing with the disease (unpublished work). He regards them weak, unstable and even non-specific, and is unable to assign them a value other than doubtful (±). He is aware that they occur in treated cases of syphilis.

TWO TYPES OF POSITIVE SERA DIFFERENTIATED BY THE USE OF A PLAIN AND A CHOLESTERINIZED HEART EXTRACT. TERMS STRONGLY POSITIVE AND POSITIVE PROPOSED TO DISTINGUISH THEM.

It is well known that a plain alcoholic heart extract fixes less complement with a positive serum than a cholesterinized heart extract. The writers find that it fixes complement with a certain fraction of positive sera only. This difference is of such an order as to justify a division of the positive sera into two classes, (i) those which fix complement with plain heart extract and (ii) those which fix complement with cholesterinized heart extract only. In a series of cases from the Carmichael Hospital for Tropical Diseases, Calcutta, studied especially for another purpose, the following figures have so far been obtained:—

	Indians.	Europeans, Anglo-Indians, and others.
Positive with plain heart extract ..	6	1
Positive with cholesterinized heart extract.	38	14
Weakly positive	1	1
Doubtful	101	32
Anti-complementary	6	2
Negative	148	100
TOTAL ..	300	150

In bloods received from venereal clinics the number of the sera fixing complement with the plain heart extract is, of course, greater.

When pooled in lots of six such sera will continue fixing complement with the plain heart extract on further dilution. Dilutions of 1 in 10 and 1 in 20 are found effective. With a cholesterinized heart extract their reaction is of a much higher order. Dilutions of 1 in 50, 1 in 100, and 1 in 200 are found effective. It is evident that the usual 1 in 5 dilutions of such sera will be fully positive irrespective of the quality of the antigen or the quantity of the complement, in spite of any irregularity in the titration of the reagents, and according to any method one chooses to employ. Therein lies the futility of using them, in the usual dilution of 1 in 5, as controls in a day's work or in testing new stocks of antigens.

The sera reacting with only the cholesterinized heart extract are in a different class. Their reaction weakens on further dilution rapidly. Most of them will react in 1 in 10, many in 1 in 20, some in 1 in 30, a few in 1 in 40, and only occasionally one in 1 in 50.

It is proposed to call the sera completely inhibiting hæmolysis with the plain heart extract strongly positive (+ + +) and the sera completely inhibiting

hæmolysis with only cholesterinized heart extract merely positive (+ +). The serum in both cases is diluted 1 in 5. The M. H. D. of the complement is 4 in the case of the plain heart extract and 5 in the case of the cholesterinized heart extract. The difference in the dose of the complement depends upon the fact that while the plain heart extract is not anti-complementary at all the cholesterinized heart extract is anti-complementary to the extent of 1 M. H. D. The *functioning* dose of the complement, therefore, is the same.

Judged by the relation between the usual and the minimal quantity of the serum used in obtaining a fixation of complement, strongly positive sera of the writers are of about the same order as very strongly positive sera of Kolmer (1929).

Iyengar (1919) at Kasauli introduced into the Method Number 4 another tube with 8 M. H. D. of complement. The positive reaction indicated by these tubes (with 3, 5, and 8 M. H. D. of complement) is also designated + + +. From what has been said on the titre of the positive sera with respect to their reactions with the uncholesterinized and cholesterinized antigens, it is evident that the distinction so obtained between a + + + and a + + reaction is not of the same order as the one advocated in the present communication.

The difference between a 'strongly positive' and a 'positive' serum (as defined by the writers) in fixing complement with cholesterinized antigen is not one of quantity only. There are some positive sera (+ +) whose end point with cholesterinized antigen is the same as that of certain strongly positive sera (+ + +), yet they fix no complement with uncholesterinized antigen (plain heart extract) in a dilution of 1 in 5. There is, thus, a difference of quality also.

USE OF A STRONGLY POSITIVE SERUM AS A CONTROL IN THE DAY'S WORK AND IN TESTING NEW STOCK OF ANTIGEN.

TITRATED CONTROLS.

Six strongly positive sera are pooled and filtered through a Seitz filter. From the pooled serum dilutions of 1 in 10, 1 in 20, 1 in 50, 1 in 100, and 1 in 200 are made. The dilutions 1 in 10, 1 in 20, and 1 in 50 are put up with 4 M. H. D. of complement and the plain heart extract, already in use, diluted 1 in 15 with saline. The dilutions 1 in 50, 1 in 100, and 1 in 200 are put up with 3 M. H. D. and 5 M. H. D. of complement and the usual cholesterinized heart extract antigen prepared in accordance with method number four. The dilutions put up with the plain heart extract will show complete inhibition of lysis in 1 in 10 or 1 in 20. There should be no inhibition with 1 in 50. If there is, the serum is tested in higher dilutions on the next working day. Generally a complete inhibition will be found only with the 1 in 10 or 1 in 20 dilution. Then it will be found that the corresponding five-fold or tenfold dilutions (1 in 50, 1 in 100 or 1 in 200) show a complete inhibition of lysis with the cholesterinized heart extract antigen already in use. These dilutions, now, will provide controls for the day's work and for testing new stock of antigen at the stage of plain heart extracts.

These titrations are done on a day when the titre of the complement and its reaction with the antigen already in use are optimal. On another day of optimal reactions further adjustment may be made and safe limits of dilutions giving the required reactions determined.

For testing heart extract it is an advantage to go to the extreme limit of fixation so that some extracts may yield a full fixation, others a trace of lysis and yet others a plus-minus reaction. The heart extracts yielding a full fixation are selected and others rejected. The usual dilutions of the serum used are 1 in 10, 1 in 15, 1 in 20, or 1 in 25.

For the day's work two dilutions are necessary, one giving a full fixation and the other a plus-minus reaction, with the cholesterinized antigen and the lower of the two doses of the complement. These dilutions are put up as controls instead of the known positive and weakly positive sera left over from the previous day's work. The usual dilutions are 1 in 50 and 1 in 100 or 1 in 100 and 1 in 200. Occasionally they may be 1 in 25 and 1 in 50.

The pooled, filtered, and titrated serum is kept on the bottom shelf of a refrigerator, as such in a corked tube, dried on a thick filter-paper (0.1 c.c. on half a square, 20 mm. \times 20 mm., of Whatman filter-paper, such as is used in blood work) or diluted 1 in 10 in a stoppered bottle with saline containing 0.25 per cent phenol. It keeps well. It is not re-heated before use.

The serum kept as such becomes anti-complementary after some weeks and may not be suitable for testing heart extract if the required dilution of it does not yield a complete lysis in the serum control. For providing controls for the day's work it will be available for months. One in 25, 1 in 50, 1 in 100, and 1 in 200 dilutions of it will not be found anti-complementary in the serum controls. A sediment appearing in the preserved serum lowers its titre.

The sera stored dried on filter-paper, in a bottle, kept in a desiccator which is left on the lower shelf of a refrigerator, stay effective for over a year at least.

The serum diluted 1 in 10 with phenolized saline is the easiest to prepare, to use and to keep. The bottle is taken out of the refrigerator, shaken and the required quantity of the fluid removed with a clean pipette. Phenol in it does not interfere with the fixation of the complement. Its titre may fall once with the appearance of a sediment. Later, it remains constant at a lower level.

The advantages of using a pooled strongly positive serum in the controls are that:—

- (i) it is obtained from cases which are least likely to give a false positive reaction and are, therefore, most likely to be cases of syphilis.
- (ii) its high titre makes graded dilutions, with wide gaps between them, possible. From them titrated controls can be obtained.

The only consideration against the use of such a serum is that in a laboratory not called upon to do many Wassermann reactions enough sera of this class may not be available. They can be obtained from elsewhere, titrated locally for any depreciation in transit and stored.

Working with the titrated controls the writers have been studying the capacity of the complement for being fixed in relation to its type, titre, and dose, keeping the antigen constant, and the fixing power of the antigen in relation to its type, titre, and dose, keeping the complement constant. These items will be discussed in future communications. The titrated controls employed, in experiments conducted alongside one's daily routine can and do supply as much information on these and

allied items as critical analysis of mass reactions involving thousands of cases. In fact they supply better information in as much as the experiments undertaken are repeatable and are repeated.

PRESUMABLE SIGNIFICANCE OF A STRONGLY POSITIVE (+ + +) REACTION
IN THE DIAGNOSIS OF SYPHILIS.

A strongly positive reaction, as defined, may be and probably is merely an emphasized positive reaction. In that case its significance will be greater than that of an ordinary positive reaction. Further, it is likely that it disappears earlier than the ordinary positive reaction. That is why it is found more often in cases from venereal clinics than in cases from general hospitals. The following two cases from the series mentioned above, however, go counter to such assumptions:—

CASE 1. G. B., aged 9 years, an American boy, admitted to the Carmichael Hospital for Tropical Diseases, Calcutta, on 29th July, 1937, suffering from low pyrexia of two to three months' duration. Liver enlarged and firm. No other abnormality. Only positive finding a strongly positive W. R. No suggestive parental history. Father, mother, and brothers normal and their W. R. negative. No scars. Very good home. No possibility of interference by servants.

A course of eight bismuth injections given. Improvement after the second injection; pyrexia disappeared, weight increased, and size of liver decreased. Patient discharged on 15th September, 1937, with advice to continue treatment.

Examined again on 8th March, 1938, after eight more injections of bismuth. Liver still palpable and firm. General health very good. W. R. completely negative.

Was one dealing with visceral syphilis modified in the tropics or with a tropical disease so far unidentified? One inclined in the beginning to the first view but now one inclines to the second. In less than a year 11 such cases have been collected with a positive or doubtful W. R. and no presumption of syphilis. The W. R. of the patient under reference was + + +. Others gave weaker reactions. Total number of beds in the hospital is 106. Total number of patients admitted last year was 1,026.

The patient in spite of his strongly positive reaction was in all probability not a case of syphilis.

CASE 2. M., aged 50 years, a Mohammedan male, admitted to the hospital on 9th March, 1938, suffering from dehydration after a severe attack of diarrhoea. History of previous similar attacks. Improvement in hospital rapid. W. R. + + + on 11th March, 1938. Repeated on 1st April, 1938, and found + + ±. Scar on penis present. History of syphilitic infection 22 years previously.

The patient gave a strong reaction 22 years after infection.

THE DANGER OF FALSE REACTIONS.

Working with the titrated positive controls, kept constant for months together and expected to give readings of + ± and ± the writers find that the risk of missing a weakly positive reaction, following method number four, must be negligible indeed, if it exists at all. They have worked for over a year with controls obtained from lots of six pooled sera. The dilution of the pooled sera giving a + ± reaction with the cholesterinized antigen has been of the order of 1 in 200 to 1 in 25. The dilution giving a ± reaction has never been below 1 in 50. Changes in the type of the complement, prolonged incubation after adding red blood cells such as occurs

in Calcutta in the hot weather at room temperature, and the possible prolonged action of the complement when the racks are left in a refrigerator overnight, have never succeeded in altering the expected reactions of the controls. A $++$ control has remained $++$. A \pm control has only varied with regard to the degree of lysis. It has never become negative.

Dilutions made from positive sera ($++$, giving reaction with cholesterinized antigen only) have been equally satisfactory. A serum of this class must as a rule be diluted to considerably more than a 1 in 10 before its reaction changes.

The risk of reading a reaction plus instead of plus-minus, on the other hand, is considerable until the red blood cells have gravitated leaving on top clear fluid in which traces of lysis can be definitely excluded.

A glance at the figures giving proportion of strongly positive, positive, doubtful and negative reactions in an unselected hospital population will show that the positive rate of the writers is lower than that of previous workers on the subject. The point, however, will not be discussed in the present communication.

SUMMARY.

1. Wassermann positive sera giving a $++$ reaction (Method No. 4 of the British Medical Research Council) in the usual dilution of 1 in 5 may give the same reaction even when diluted 1 in 200. Such sera are faulty as controls in a day's work and utterly useless in testing new stocks of antigen.

2. It is possible to separate the positive sera into strongly positive ($+++$) and positive ($++$) by testing them with uncholesterinized and cholesterinized heart extract. Only strongly positive sera react with the former. They differ from positive sera quantitatively as well as qualitatively.

3. Strongly positive sera can be pooled, titrated, and preserved. Known dilutions made from the pooled serum can be used as controls in a day's work and in testing new stocks of antigens. They are titrated controls.

4. Problems needing critical analysis of mass reactions involving blood reactions of thousands of cases can also be solved by using titrated controls as constants.

5. A strongly positive reaction does not, at least under tropical conditions, always strengthen a presumption of syphilis, though usually it does so. A strongly positive reaction probably occurs in secondary syphilis and in the early stage of infection. It may, however, be found 20 years after infection.

6. As shown by titrated controls slight variations in procedure, intentional and unintentional, are not likely to give false negative readings. There is always a risk, however, of obtaining a false positive reading, unless the possibility of traces of lysis in the tubes is definitely excluded.

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EXPERIMENTAL STUDIES TO GUIDE THE SELECTION OF THE MOST SATISFACTORY TYPE OF ANTI-WELCHII SERUM FOR THE PREVENTION AND TREATMENT OF GAS GANGRENE IN MAN.

BY

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THE problem of the proper selection of the most satisfactory type of anti-welchii serum is a very complex one. The disease is due to the invasive power of the organisms, as well as to the action of toxins produced by them. Hence it appears that a serum to be of use should be able not only to neutralize the specific toxins, but also to protect against the invasive action of the organisms. Or, in other words, a serum to be of use in protecting against a natural infection in man must contain antitoxins and also antibacterial antibodies against the surface antigens of all the possible types of *Cl. welchii*.

A consideration of the mode of development of gas gangrene in man raises some doubt regarding such a conclusion. As quoted by Topley and Wilson (1936) from Special Report (Medical Research Council, 1919) 'The disease begins, not when a wound has become infected with the pathogenic anærobes, but from the moment when a group of these bacteria have been enabled to surround themselves with a toxin sufficiently concentrated to abolish the local defences of the tissues'. If so, then a sufficient concentration of antitoxin present in and around the site of infection should at least be able to prevent the beginning of the disease, if not cure it when already begun, by the neutralization of the toxins as soon as they are formed, thus allowing the local defence mechanism to deal with the infection. Antibacterial antibodies alone do not seem to be useful for this purpose.

On the other hand, Robertson and Felix (1930) and Henderson (1934, 1935, 1937) have found that a specific antibacterial immunity can be established against experimental infection with *Cl. septicæ*. They prepared protective sera in rabbits against the heat-stable 'O' antigen and (Henderson, 1935, 1937) also against a formalized unheated culture and showed that such sera were more powerful than an antitoxic serum in preventing the development of gas gangrene induced in experimental animals (guinea-pigs and mice) by the inoculation of 'activated'

spores. Though they have worked with *Cl. septicus*, the result appears to have some bearing on the present problem, because the mode of disease production is the same with *Cl. septicus* and *Cl. welchii*. The disease syndromes produced by them are also similar.

This conflict of ideas naturally raises the question, whether antitoxic or antibacterial serum alone, or a combination of both, should be used for the prevention and treatment of gas gangrene in man. A tentative answer to this question has been attempted in this paper, by comparing the protective powers of the two types of sera against an experimental infection of laboratory animals with *Cl. welchii*.

That is not the only problem. In the preparation of a suitable antitoxin, one has to consider the fact that toxin-antitoxin neutralization experiments made in relation to classical *Cl. welchii* and other closely resembling organisms isolated from diseased sheep and called the lamb dysentery bacillus (or agni variety of *Cl. welchii*), *Cl. paludis*, and *Cl. ovitoxicus*, have shown that these organisms produce exotoxins which clearly differ from one another in antigenicity and some other characters (Mason *et al.*, 1931; Glenney *et al.*, 1933). On the basis of the factors named W, X, and Z, present in the toxins liberated by those different organisms, Wilsdon (1931, 1933) has classified the strains of *Cl. welchii* into types A, B, C, and D, corresponding respectively to the *welchii* lamb dysentery, *paludis*, and *ovitoxicus* types of organism. Toxin of the type A contains the factor W, of the type B the factors WXZ, of the type C the factors WZ, and of the type D the factors WX.

Weinberg and Guillaumie (1937) have shown that with the exception of toxins of types B and C, either of which produces both the antitoxin B and the antitoxin C in horses, almost in equal amounts, the other antigens produce only the corresponding antibodies. So that in the preparation of a 'holoserum' (the term proposed by Weinberg to indicate a serum prepared against the organisms of the same species, whereas the term 'polyvalent serum' should be used to indicate one prepared against the organisms of different species) either a mixture of toxins of types A, B, and D or that of toxins of types A, C, and D may be used. A serum prepared against a strain of classical *Cl. welchii* contains only the antitoxin A.

Now, it has been shown by Borthwick (1935) that protection by monovalent sera, against experimental infections with different types, depended on the ability of the serum to neutralize the homologous toxin. An exception to this general rule was the power of the serum prepared against toxin of the type A and the commercial anti-welchii serum to protect against infections by organisms not only of the type A, but also of the type D, although the protecting sera were able to neutralize toxin of the type A only. He stressed the practical importance of the inability of the commercial antisera to protect against infections of types B and C, for he considered that there was no theoretical reason why the types B and C should not also produce disease in man, as toxins of those types also contain some of the W constituent, which is the only factor present in toxin of the type A, the recognized causal agent of gas gangrene in man.

Should the polyvalent antitoxin be prepared and used instead of a monovalent anti-welchii serum containing antitoxin A only? A definite answer to this question cannot be given at present. If it is proved beyond doubt that the type A organisms are the only causal agents of the disease in man, then the use of polyvalent antitoxin

becomes superfluous. However, one thing has been made clear by Weinberg and Guillaumie (*loc. cit.*), that the power of the polyvalent antitoxin containing antitoxins A, B, C, and D, in protecting against an experimental infection with the type A organisms, is the same as that of the monovalent anti-welchii serum. Hence it appears to be of no advantage to use a polyvalent serum if organisms of types other than the type A are not recognized to be causal agents of the disease in man.

Another fact deserving consideration is that toxin of the type A shows three different biological effects: hæmolysis, a necrotic effect, and a pharmacological effect. All of them are of importance in the pathogenesis of the disease in man. Hæmolysis is responsible for anæmia. The necrotic effect helps the invasion of the tissues by the organisms, as the lowered oxidation-reduction potential in the necrosed areas, whose normal oxygen supply is cut off owing to stoppage of the circulation in the damaged vessels of the necrosed areas, favours multiplication of these anærobic organisms, which while growing liberate more toxin to maintain the vicious circle. The pharmacological effect causes rapid death. Hence a therapeutic serum should be able to neutralize all those effects of the toxin in equal degrees, in order to offer a perfect protection against the disease in man. Do the commercial anti-welchii sera prepared in horses against the type A organisms fulfil this condition? Experiments which answer this question are described in this paper.

These are perhaps only some of the most pressing questions in the proper selection of a satisfactory type of anti-welchii serum for the prevention and cure of a natural infection in man.

The following experiments have been undertaken to bring some evidence to guide the proper selection of such a serum.

RELATIVE PROTECTIVE POWERS OF ANTITOXIC AND ANTIBACTERIAL SERA AGAINST EXPERIMENTAL INFECTION.

Technical.

Antitoxic serum was prepared in a horse at first with formol toxoid and then with pure toxin of a strain of classical *Cl. welchii*. The serum used for the experiment contained about 200 International Units of antitoxin per c.c.

Antibacterial serum was prepared in a horse at first with 18 hours' culture of *Cl. welchii* in veal-peptone broth, killed by heating at 56°C. for 30 minutes and later on with living cultures given intravenously, the final dose consisting of about 50,000 million organisms (by opacity), this being repeated till a satisfactory titre was obtained. The serum used for the experiment agglutinated a standard killed suspension of *Cl. welchii* in a dilution of 1 in 800, and also contained about 20 International Units of antitoxin per c.c.

The sera were preserved with 0.5 per cent phenol and stored at 0°C. to 2°C.

Living bacterial suspension was prepared as follows: The organisms (*Cl. welchii*) of the same strain as used for the preparation of antibacterial serum were grown in cooked meat medium for 5 hours at 37°C. The supernatants were

collected in sterile centrifuge tubes and centrifuged. The supernatants were pipetted off, the deposits re-suspended in sterile Ringer's solution, and centrifuged again. The supernatants were again pipetted off and the deposits finally suspended in Ringer's solution to match the opacity of a standard suspension of 1,000 million *B. coli* per c.c.

Repeated experiments proved that 1 c.c. of such a suspension injected in the pectoral muscles always killed a pigeon of about 300 g. weight within 24 hours.

Experimental.

A. Protection against immediate experimental infection.—The experiment was done on ten consecutive days. Each day four groups of two pigeons each were used for the experiment. The pigeons were of the same breed and nearly of the same age and weight. The same samples of antitoxic and antibacterial sera were used every day. But the bacterial suspension was prepared fresh every day, using the same stock culture for every day's subculture and following the same procedure each day.

The protective sera were injected in the pectoral muscles of the left side, one hour before injection of the bacterial suspension. Pigeons of group A were injected with 1 c.c. (200 I. U.) of antitoxic serum; those of group B with 0.5 c.c. (100 I. U.) of antitoxic serum; those of group C with 3 c.c. of antibacterial serum, also containing 60 I. U. of antitoxin; and those of group D with 1.5 c.c. of antibacterial serum, also containing 30 I. U. of antitoxin.

One hour after the injection of the protective sera, 1 c.c. of bacterial suspension was injected into the pectoral muscles of the right side of each of the pigeons.

The interval of one hour was found to be necessary even for testing the immediate protective action, as otherwise such a rapidly growing organism almost completed its action before the protective sera were absorbed to any effective extent.

The pigeons which succumbed usually died in about eight to ten hours, excepting a few which died later. Those which did not die were usually apparently healthy after 24 hours.

The result is recorded according to the survival rate in different groups of pigeons protected with different amounts of antitoxic and antibacterial sera (Table I).

B. Protection against delayed experimental infection.—For this purpose two groups of five pigeons in each group were used. Pigeons of group A were injected with 0.4 c.c. (80 I. U.) of antitoxic serum and those of group B were injected with 1.0 c.c. antibacterial serum also containing 20 I. U. of antitoxin per c.c. in the pectoral muscles of the left side. On the fourth day after injection of the protective sera, each one of them was injected with 2 c.c. of bacterial suspension prepared from the same stock as used in the previous experiment and following the same procedure as before.

The experiment was repeated.

TABLE I.

The relative value of antitoxic and antibacterial sera in the protection against an immediate experimental infection.

Day.	Group A (2 pigeons) protected with 1 c.c. antitoxic serum.	Group B (2 pigeons) protected with 0.5 c.c. antitoxic serum.	Group C (2 pigeons) protected with 3 c.c. antibacterial serum.	Group D (2 pigeons) protected with 1.5 c.c. antibacterial serum.
1st ..	2	2	1	0
2nd ..	2	1	2	1
3rd ..	2	1	2	0
4th ..	2	2	1	0
5th ..	1	1	0	0
6th ..	2	2	1	0
7th ..	2	1	2	1
8th ..	2	2	1	0
9th ..	2	2	1	0
10th ..	2	1	2	0
Total survival ..	19	15	13	2

The result is recorded in survival rate in two groups of pigeons protected with antitoxic and antibacterial sera respectively (Table II) :—

TABLE II.

The relative value of antitoxic and antibacterial sera in the protection against a delayed experimental infection.

Day.	Group A (5 pigeons) protected with 0.4 c.c. antitoxic serum.	Group B (5 pigeons) protected with 1.0 c.c. antibacterial serum.
1st ..	5	0
2nd ..	5	1
Total survival ..	10	1

The results of both these experiments signify that in a suitable dose antitoxic serum alone can protect an animal against both an immediate and a delayed experimental infection; and that the protection offered by an antitoxic serum is significantly better than that by an antibacterial serum, when compared volume to volume. The interpretation of a better protection offered by higher doses of antibacterial serum used in these experiments is rendered difficult owing to the simultaneous effect of moderate amounts of antitoxin present in the sera.

How far these results are applicable in the protection against a natural infection in man cannot be settled by a trial of these sera in a few scattered cases of peace-time infections. Also the result obtained by a trial of these sera in protection against peace-time infections may not be applicable for controlling war-time infections. Peace-time and war-time infections appear to be of a different nature, as has been discussed by Aschoff (1938); their means of control may also require to be different.

POWER OF THE COMMERCIAL ANTI-WELCHII SERUM TO NEUTRALIZE ALL THE THREE EFFECTS OF THE TYPE A TOXIN EQUALLY.

The accepted methods for the particular type of toxin-antitoxin titrations are mice protection tests and intradermal tests in guinea-pigs (Hartley and White, 1935; Walbum and Reymann, 1935). In the present series of experiments, sheep cells were used for the study of the hæmolytic effect; rabbits were used for the studies on the dermo-necrotic effect; and pigeons for the pharmacological effect of toxin of the type A, and the power of the commercial sera to neutralize those effects.

Technical.

Preparation of toxin-broth.—Type A *Cl. welchii* was grown in veal-peptone-broth containing a few pieces of ox-liver for 18 hours at 37°C.; the broth was filtered and precipitated with ammonium sulphate, the precipitate was re-dissolved in distilled water and dialysed against running tap-water for three days at 0°C. to 2°C. The partially purified and concentrated toxin-broth was stored in sealed ampoules in the ice-chest, after filtration through Seitz E. K. pads. No preservative was used. In this condition, the biological properties of the toxin-broth remained fairly constant over a long period.

Determination of the test doses of the toxin.—The L_H , L_T , and L_+ doses of the toxin representing respectively the residual hæmolytic, necrotic, and pharmacological effects of the toxin, were determined by the usual methods against a standard serum.

Preparation of anti-welchii serum.—Increasing doses of formal toxoid and later on unaltered toxin injected subcutaneously with simultaneous intravenous injection of heat killed and later on living cultures of the type A *Cl. welchii* were used for the preparation of anti-welchii serum in the horses. Horses were bled when satisfactory titre were obtained and plasma from a number of horses was concentrated and purified in a lot by the method of Gibson and Banzhaf (1910).

Experimental.

Different lots of concentrated anti-welchii sera were titrated against L_h , L_r , and L_+ doses of the stock toxin. The same technique as used for the determination of those doses of the toxin against a standard serum was used, keeping the toxin constant and varying the amounts of serum. The result is expressed in units of antitoxin, neutralizing the particular effect of toxin against which a serum is titrated, present per c.c. of the concentrated serum of each lot (Table III):—

TABLE III.

Comparison of the power of commercial anti-welchii sera in neutralizing the various toxin effects.

Serum lot number.	Units antitoxin against hæmolytic effect.	Units antitoxin against necrotic effect.	Units antitoxin against pharmacological effect.
1	800	800	750
2	1,000	900	1,000
3	750	800	750
4	600	700	600
5	800	850	850
6	850	900	800

Hence the ability of each of the six lots of serum to neutralize the three different effects of toxin of the type A was found to be almost equal. It is a common experience that the same horse reacts differently to different antigens; as, for instance, Weinberg and Guillaumie (*loc. cit.*) observed that the injection of a mixture of toxins of the different types of *Cl. welchii* in equal proportion evoked different grades of response against the various toxins in the same horse. So, in this particular case, had the different biological effects of toxin of the type A been due to different antigenic components, it might have been expected that in each lot of serum a difference in the power to neutralize each different effect of the toxin would have been demonstrated. Moreover, toxins of different lots, prepared at different times, were used for immunizing each horse; if they contained a number of antigens in each lot, it is quite natural to suppose that their composite antigenic

properties were different each time. So, different degrees of stimulus, by each single antigenic component of toxin, present in different amounts, at different times, should have evoked different grades of response against each component at different times. Or, in other words, different titres of neutralizing substances against each of the effects—had they been due to so many different antigens—should have been expected. But it was not so. Therefore, it may be concluded that the different biological effects of the toxin of the type A are due to one antigenic substance only, so that all of them can be neutralized by an antibody against any one of them.

DISCUSSION.

Anti-gas gangrene serum on the market to-day consists of anti-welchii, anti-vibrio septique, anti-œdematiens, and also anti-histolyticus serum—the protective power of each component being measured in units of antitoxin, which are internationally accepted. These sera are prepared individually and then mixed together, thus making the total volume of the serum necessarily a big one.

If, as a result of experimental studies, it is found necessary to add to the present serum antibacterial sera prepared against each organism in a suitable way, it will increase the bulk more if, as is perhaps quite likely, these have to be prepared separately. And the climax will be reached if, to this bulk, it is found necessary to add the antitoxins against the other types of *Cl. welchii*, as most possibly these also will have to be prepared separately. The reasons for that are twofold. Firstly, as according to Weinberg and Guillaumie (*loc. cit.*) simultaneous injection in equal amounts of toxins of the types A, B, and D, necessary for the preparation of a polyvalent antitoxin, produces different and varying titres of antitoxins in the same horse at different times, it seems to be necessary to prepare each antitoxin in a different horse, in order to maintain a uniform proportion of each antitoxin in the mixture prepared for use in man. Secondly, according to the observations of Glenny and Waddington (1926, 1928), that in the production of antitoxins, the simultaneous injection of several antigens may considerably reduce the titre of antitoxin obtained, toxins of different antigenic types should not be injected simultaneously lest one of the antigens present in small amounts be 'crowded out'.

This prohibitive bulk may render the use of anti-gas gangrene serum in the prophylaxis and therapy of the disease in man difficult. It is impossible to foretell which particular type of organism may invade a man exposed to the risk; nor is it easy, in the present state of our knowledge, to find out definitely the type of the organism causing the disease in man during the short duration of the disease. Under these circumstances there appears to be no alternative but to use a mixture of all types of sera, irrespective of its bulk, if in field trials all of the components of the mixture prove to be necessary for offering the best protection against the disease in man. But if some of them are found to be unnecessary, as, for instance, antibacterial sera prove to offer no better protection when added to the mixture, or the types B, C, and D of *Cl. welchii* are not recognized to be causal agents of gas gangrene in man, after extensive field trials, then the present anti-gas gangrene serum may prove to be sufficient, provided that no other fresh organisms are incriminated.

It is thus to be clearly understood that carefully devised field experiments alone may settle the issue.

SUMMARY.

Experiments are described, the results of which show that:—

1. In a suitable dose, antitoxic anti-welchii serum can protect an animal against both an immediate and a delayed experimental infection.

2. The protection afforded by an antitoxic serum is significantly better than that by an antibacterial serum, when compared volume to volume.

3. An antitoxic serum prepared against the toxin of type A *Cl. welchii* can neutralize all the biological effects of the toxin in equal degrees, namely, its hæmolytic, necrotic, and pharmacological effects.

The problem of the proper selection of the most satisfactory type of anti-gas gangrene serum for use in man is discussed.

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THE BASAL METABOLISM OF INDIAN AND EUROPEAN MEN ON THE NILGIRI HILLS (S. INDIA).

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ORR (1938) has very recently pointed out that the trend of investigations on the qualitative side of nutrition problems has overshadowed the quantitative, so that there is the fear of belittling the importance of energy requirements. The Technical Commission of the League of Nations (1936) has also recommended new standards for 'standard metabolism'. The determination of the basal metabolism is the starting point for calculations of the energy requirements of an individual. So far as the European races are concerned the standards have been determined, e.g., by Harris-Benedict and Aub-duBois in America, and by Dreyer in England. Investigations in the tropics by several workers [first by de Almeida (1920) in Brazil, and recently by Mason (1934) and Mason and Benedict (1931) in India, to mention only two] show generally a lowered basal metabolic rate. Two separate factors might be responsible for these low values, namely, the climatic and the racial. An opportunity occurred in Coonoor to determine the basal metabolism on Indians and Europeans to elucidate the two points in regard to the energy output.

Previous work in India is summarized in Table I given below.

Subjects of this investigation.—Twenty-six Indian men, mostly laboratory workers and assistants, and medical men are classed into two groups. The first 17 are residents on the hills (in Coonoor, 6,000 feet above sea-level) for not less than three years. The last nine of the 26 form group II, who were resident in Coonoor for one and a half to two months. The Indian group consisted predominantly of Telugus and Tamils with a few North Indian Hindus. The majority of the Indians are vegetarians, and even the few non-vegetarians partake of meat very sparingly. The ages ranged between 22 and 44 years with one aged 52, the average being 31 years.

The European group consists of 20 subjects, the first two are from the laboratory staff, the remaining 18 being medical orderlies in a Military Hospital near Coonoor (Wellington). In this group the ages varied between 21 and 27 years with one aged 39. The average age was 25 years. The hospital orderlies were on the

TABLE I.
Showing previous work in India.

Year.	Name of worker and place of observation.	Apparatus employed.	Subjects.	Number of subjects studied.	Age in years.	Deviation Aub-duBois standard, per cent.
1927	Sokhey (Bombay) ..	Tissot's	Indian medical students	15	19-30	-10 to -23
1931	Banerji (Lucknow) ..	Benedict's	Indian prisoners	145	20-45	- 6.9
1931	Mukherjee and Gupta (Calcutta) ..	Douglas' bag	Bengalee young men (college students).	18	20-29	-13.8
1931	Mason and Benedict (Madras) ..	Benedict's	South Indian college women.	54	17-31	-17.2
1932	Krishnan and Vareed (Madras) ..	Benedict-Roth's	Male medical students	54	18-25	-12.0
1936	Rahman (Hyderabad) ..	Sanborn's	Medical students of Hyderabad.	32	19-32	- 8.7
1938	Wilson and Roy (Calcutta) ..	Douglas' bag	Schoolboys (Hindu and Mohammedan).	62	6-16	- 9.7 to -19.5

hills for five months and had seen service in India for not less than one year. One of the laboratory staff was in Coonoor for only two and a half months since arrival from England.

The metabolism of the Indians investigated can thus be compared with that of Europeans, under identical climatic conditions, to throw light on any racial factor affecting basal metabolism.

Technique.—A Benedict-Roth metabolism apparatus with rubber-flutter valves, and kymograph equipment (from W. E. Collins, Boston) was used. By special request, a millimetre scale was fixed to the upright, so that the excursions of the pointer attached to the counterpoise of the spirometer bell could be read off on the scale. Robertson (1937) has shown that the Benedict-Roth apparatus is as accurate as the open methods, and it has the further advantage in that the test could be accomplished more rapidly, and that there is a means of checking for a leak as the test proceeds. He has shown also that the assumption of a respiratory quotient of 0.82 in the calculations does not introduce any error, rather it is to be regarded as a correction.

The soda lime used to absorb carbon dioxide was frequently dried, and one filling of the can was never used for more than 70 tests. Rubber mouthpiece and noseclip were used.

All the tests were done between 7 A.M. and 9 A.M. during the months of October, November, and December 1937, and January, February, and March 1938; that is during the winter and spring seasons. The average temperature of the room and humidity are given below :—

Month, 1937.	Temperature, °C.	Humidity, per cent.	Month, 1938.	Temperature, °C.	Humidity, per cent.
October ..	18.3	78	January ..	16.7	70
November ..	17.5	80	February ..	16.7	73
December ..	17.3	75	March ..	17.2	67

The nature of the test was explained to each subject before carrying it out on him. In the case of the laboratory staff and assistants and doctors, the tests were made in a quiet room on the ground floor of the laboratory. Those who were within five minutes' walk to the room came to the experimental room walking in the morning with an empty stomach (at least 12 hours after the previous meal) and lay on the bed without movement, covered with a blanket for 30 to 60 minutes. In the Military Hospital at Wellington the subjects had their high tea at 5 P.M. to 6 P.M., slept in a room in the hospital itself, and as soon as they awoke in the morning walked into another room in the hospital where the apparatus was kept and had a preliminary rest of half to one hour. In Coonoor, only one subject a day was tested up to Nos. 1 to 17. The subjects, Nos. 18 to 26, were tested two a day. In the Military Hospital in Wellington two subjects were tested every day, one taking preliminary rest in bed while the other was being tested. On each subject three trials lasting 9 to 10 minutes each were done,

the reading of the pointer on the scale read off every minute, while the pen traced the respiration curve on the kymograph drum (with a time recorder). During the course of each test the weight was placed on the spirometer bell for two minutes to make sure there was no leakage. At least three pulse counts were made, using a stop-watch during the course of a test. The readings of the pointer on the millimetre scale were plotted against the number of minutes and a straight line to pass through the majority of points was drawn and the difference in millimetres for eight minutes were used for the calculation of oxygen consumption (1 mm. drop of the spirometer bell = 20.73 c.c. of oxygen, assuming R. Q. = 0.82 and 4.825 calories the heat equivalent of one litre of oxygen consumed).

The kymograph records were used to calculate directly the output of calories per hour (the calibration of the apparatus is such, that the trend of the slope of the curve measured in millimetres in a six-minute spacing is equivalent, for the same number, to calories per hour). The slope is determined by using a celluloid ruler and drawing a straight line to pass through as many points as possible on the lower border of the curve. The tests were repeated on at least two days in each case, and there were some on whom the tests had to be repeated on three to four days to get the lowest metabolic rate. The investigation was considered satisfactory when the values for oxygen consumption of two tests on the same day for a subject agreed within 3 c.c. to 4 c.c., and when these calculated as calories per hour approximated within 5 per cent the calories calculated from the kymographic record. The lowest value of oxygen consumption and heat output per hour have been used to work out the deviations from the predicted standards. This is to be preferred to the averaging of values. Three hundred and eighteen tests were carried out in all on 46 men.

PHYSICAL CHARACTERISTICS OF SUBJECTS.

Table II shows some of the physical measurements taken on the subjects.

In the Indians, the average height was 168 centimetres, sitting height (following Dreyer's direction) 84 centimetres, weight without shoes (weight of clothing deducted) with an empty stomach 56.0 kilograms. The surface area was 1.61 square metres on the average. The corresponding figures for the European group are 173 centimetres, 89 centimetres, 62.2 kilograms, and 1.73 square metres. Thus the European group is apparently of slightly larger physique.

Pelidisi.—Pirquet's pelidisi is calculated according to the formula—

$$3 \sqrt{\frac{10 \times \text{weight in grammes}}{\text{sitting height in cm.}}} \text{ multiplied by } 100.$$
 It is considered that for Europeans a figure somewhere near 97 or 98 is normal. This index is supposed to represent the state of nutrition, values below 90 show a low state of nutrition or very thin subjects and that over 100 represents a fat person. In the subjects of the present study, among the Indians only two showed a pelidisi of less than 90, the other 24 had the value between 91 and 112, the average for the whole group being 98. No European subject has a pelidisi less than 91, the range was 91 to 115, the average of 20 being 97, so the state of nutrition evaluated according to this index in both Indians and Europeans agrees well and can be taken as normal.

Physical characteristics of subjects studied.

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INDIANS.										EUROPEANS.								
Subject number.	Age in years.	Height in centi- metres.	Sitting height in centimetres.	Weight in kilo- grams.	Surface area in square metres.	Pelidisi.	Subject number.	Age in years.	Height in centi- metres.	Sitting height in centimetres.	Weight in kilo- grams.	Surface area in square metres.	Pelidisi.					
1	36	169	85	61.3	1.70	100	1	27	183	91	64.0	1.82	95					
2	23	159	77	43.8	1.42	99	2	39	176	89	71.3	1.86	100					
3	31	166	82	54.3	1.58	100	3	24	170	88	68.2	1.77	100					
4	30	168	84	49.3	1.53	94	4	21	170	88	62.2	1.70	97					
5	26	167	87	46.8	1.50	89	5	26	181	89	65.0	1.82	97					
6	31	164	85	51.8	1.54	95	6	23	168	91	55.8	1.62	91					
7	26	169	87	47.0	1.51	89	7	27	166	88	65.8	1.71	99					
8	22	160	80	46.3	1.44	97	8	24	176	90	58.5	1.70	93					
9	30	173	82	52.2	1.61	98	9	23	173	91	60.3	1.70	93					
10	35	176	86	58.5	1.71	97	10	23	174	88	61.5	1.72	97					
11	37	161	83	61.5	1.63	103	11	24	191	91	66.2	1.91	96					
12	52	162	78	66.8	1.70	112	12	22	169	86	62.5	1.70	99					
13	32	170	80	57.5	1.65	104	13	23	171	90	54.5	1.62	91					
14	23	171	88	50.8	1.58	91	14	21	170	85	52.3	1.59	95					
15	27	166	88	52.8	1.57	92	15	22	169	88	59.5	1.66	96					
16	27	162	82	52.8	1.54	102	16	25	173	89	66.2	1.77	98					
17	26	171	88	50.5	1.57	91	17	23	166	90	59.0	1.64	93					
18	23	175	87	50.5	1.62	92	18	22	175	93	71.3	1.85	96					
19	44	166	85	66.5	1.72	103	19	23	174	90	65.5	1.77	97					
20	30	181	91	61.5	1.77	94	20	27	164	84	54.7	1.57	115					
21	28	165	84	55.5	1.59	98					
22	30	164	82	71.3	1.76	109					
23	41	178	92	66.2	1.80	95					
24	29	162	82	56.2	1.58	101					
25	44	164	84	53.5	1.56	97					
26	30	171	84	70.5	1.80	106					
Average { Group I (1-17) Group II (18-26) Group III (27-40)							}											
							30	167	84	53.2	1.58	97	25	173	89	62.2	1.73	97
							33	170	86	61.3	1.69	99
							31	168	84	56.0	1.61	98

SOME PHYSIOLOGICAL CHARACTERISTICS.

Data were collected on the subjects for month temperature, pulse rate, respiration rate, and oxygen consumption. These are presented in Tables III and IV :—

TABLE III.

Physiological characteristics of subjects studied.

Indians.

Subject number.	Average month temperature, °F.	PULSE RATE PER MINUTE.	RESPIRATIONS PER MINUTE.	OXYGEN CONSUMPTION PER MINUTE IN C.C.	
		Average.	Average.	(i)	(ii)
1	98.1	69	18	191	190
2	97.8	58	17	180	177
3	97.4	66	18	196	192
4	96.7	56	23	176	178
5	96.6	64	11	189	182
6	97.7	60	16	189	185
7	97.3	58	13	172	170
8	97.9	63	14	175	174
9	97.5	59	16	201	198
10	97.6	50	20	204	195
11	97.3	73	15	208	210
12	97.9	69	19	205	206
13	97.9	63	10	210	209
14	97.5	58	25	202	205
15	97.4	60	15	186	178
16	98.1	53	18	199	195
17	97.6	65	17	196	187
18	97.8	62	18	211	196
19	97.5	65	11	188	187
20	97.3	67	15	213	208
21	97.5	71	18	195	185
22	97.1	62	11	214	216
23	97.2	49	13	201	200
24	97.1	60	9	188	183
25	97.0	64	12	183	188
26	97.2	59	14	209	206
<hr/>					
Average {	Group I (1-17)	97.5	61	17	190
	Group II (18-26)	97.3	62	13	196
	1-26	97.5	62	16	192

TABLE IV.

Physiological characteristics of subjects studied.

Europeans.

Subject number.	Average mouth temperature, °F.	PULSE RATE PER MINUTE.	RESPIRATION RATE PER MINUTE.	OXYGEN CONSUMPTION PER MINUTE IN C.C.	
		Average.	Average.	(i)	(ii)
1	97.1	68	15	235	233
2	97.1	54	10	202	202
3	97.5	53	11	236	242
4	97.0	49	15	233	229
5	97.6	62	16	210	214
6	97.5	58	6	240	238
7	97.4	66	18	232	224
8	97.4	58	15	252	249
9	96.9	55	19	226	227
10	97.0	50	19	240	253
11	97.7	51	13	218	215
12	97.4	51	7	199	202
13	97.5	66	23	218	221
14	97.1	60	13	222	218
15	97.0	59	12	228	227
16	97.3	44	19	233	236
17	97.2	50	18	198	204
18	97.5	60	19	246	251
19	98.1	59	16	248	248
20	97.9	59	16	206	208
Average ..	97.4	56	15	Average of lowest value in c.c. 224	

Mouth temperature.—This was measured with a Zeal clinical thermometer under the tongue, five minutes being allowed to record the reading and the average for two or more days for each subject is given. It was found to be 97.5°F. on the average for Indians and 97.4°F. for Europeans.

Pulse.—The pulse count was taken with a stop-watch at least three times during the course of a single test. Thus for each day an average of at least nine counts were taken, the lowest for the Indians was 48 beats per minute, the highest 73. The average pulse rate was 62 per minute. Among Europeans the range was 48 to 68 with an average of 56 beats per minute. It is seen for the latter group the pulse rate is lower than for the former group. In the case of Western adult men (Harris and Benedict, 1919, quoted by Rahman, 1936) the average pulse rate in basal condition is given as 62.26 ± 6.73 . On this basis the average of 56 for the Europeans studied in Wellington approaches the lower normal limit.

Respiration rate.—The respiration counts are taken from the kymograph records, an average of three minutes from the best portion of the curve for each

test, so that the average of nine counts (of three trials) for a day are given. The average rate was 17 for the first group of Indians with variations from 10 to 25, only four had respiration rate of 20 to 25 per minute. In the second group of Indians the average was 13 per minute with variations from 9 to 18 per minute. The average for the two groups put together was 16 per minute. Among the Europeans studied there were two subjects with 6 and 7 respirations per minute. The respirations were deep. The majority had 10 to 20 per minute. The average for the group was 15. It is seen, there is no significant difference in the two races.

Oxygen consumption.—The oxygen consumption for three separate tests on a subject in a day was calculated, and when at least two tests agreed within 3 c.c. to 4 c.c. it was taken as satisfactory, the lower value accepted as the one representing basal consumption of oxygen. In almost all the cases this was found to agree within 5 per cent, with the heat output calculated according to the value on the kymograph record. The tests were repeated on a second day or sometimes a third and fourth day and satisfactory results only were taken. In the last two columns of Table III, the lowest oxygen consumption per minute by Indians on two days are given. Except in the case of five subjects, the values on two days agree within 5 c.c. The oxygen consumption on the day it is lowest has been averaged for the group, and it is found to be 190 c.c. per minute for the first group of Indians, 196 c.c. per minute for the second group and for all the 26 was 192 c.c. per minute.

The variation in the oxygen consumption for the two groups is also different. In the first group it was 170 c.c. to 209 c.c. and in the second 183 c.c. to 214 c.c. This apparent divergence narrows down when it is remembered that the average surface area of the first group is 1.58 square metres and for the latter 1.69 square metres. Among the Europeans the variation was found to be 198 c.c. to 253 c.c. per minute with an average of 224 c.c. per minute (Table IV, last two columns). The average surface area for this group was found to be 1.73 square metres, hence apart from the difference due to the average size of the group, the European consumes more oxygen per minute than the Indian. The possible factors for this will be dealt with later.

HEAT EXPENDITURE IN BASAL CONDITION.

The month the test was carried out, number of tests made, the lowest oxygen consumption, heat output in calories per 24 hours and per square metre of body surface are presented in Tables V and VI. A respiratory quotient of 0.82 was assumed and Carpenter's (1924) table was used to determine the heat output in calories from the oxygen consumption per minute. The deviations from basal metabolic prediction standards are also shown.

Heat output in calories.—In group I among Indians consisting of 17 subjects the expenditure of energy in basal condition for 24 hours was 1,181 calories to 1,452 calories with an average of 1,317 and for group II consisting of 9 subjects it was 1,271 to 1,487 calories and 1,360 calories, respectively. The average for all the 26 Indians was 1,332 calories. The significance of this is better understood when the values are calculated on the basis of per square metre of surface area (the surface area was read off from height and weight data on the Roth's metabalometric chart). For group I on this basis the average expenditure of

TABLE V.

Showing the oxygen consumption, heat output, and deviation of basal metabolism from the standards.

Indian subjects.

Month.	Subject number.	Age.	EXPERIMENTAL.		Lowest oxygen consumption per minute in c.c.	HEAT PRODUCTION PER 24 HOURS.		DEVIATION FROM STANDARDS.	
			Days.	Periods.		Total calories.	Per sq. m. calories.	Aub-duBois.	Harris-Benedict.
October 1937	1	36	2	6	190	1,320	776	-18.1	-12.6
"	2	23	4	12	177	1,230	866	-8.7	-6.0
"	3	31	2	6	192	1,334	843	-10.9	-7.0
"	4	30	3	9	176	1,223	799	-15.6	-11.5
November 1937	5	26	2	6	182	1,265	843	-11.2	-7.7
"	6	31	2	6	185	1,285	834	-12.1	-7.6
"	7	26	2	6	170	1,181	782	-17.5	-14.7
"	8	22	3	9	174	1,209	810	-11.6	-10.8
"	9	30	2	6	198	1,376	855	-10.2	-4.9
"	10	35	2	6	195	1,355	792	-16.7	-10.6
November and December 1937.									
December 1937	11	37	3	9	208	1,445	887	-6.3	-1.6
"	12	52	2	6	205	1,424	838	-7.0	-1.4
"	13	32	2	6	209	1,452	880	-7.0	-2.6
"	14	23	3	9	202	1,403	888	-6.3	-4.2
"	15	27	2	6	178	1,237	788	-16.8	-14.2
"	16	27	2	6	195	1,355	880	-7.3	-4.6
January 1938	17	26	2	6	187	1,299	827	-12.9	-9.9
"	18	23	2	6	196	1,362	841	-11.4	-8.1
October 1937	19	44	2	6	187	1,299	755	-18.5	-14.2
March 1938	20	30	2	6	208	1,445	816	-14.0	-10.5
"	21	28	2	6	185	1,285	808	-14.7	-12.3
"	22	30	2	6	214	1,487	845	-10.9	-10.7
"	23	41	2	6	200	1,390	772	-16.0	-12.6
"	24	29	2	6	183	1,271	804	-15.1	-12.6
"	25	44	3	9	183	1,271	815	-11.6	-4.1
March and April 1938.									
April 1938	26	30	2	6	206	1,431	794	-16.2	-15.3
Average {	Group I (1-17)		2	7	190	1,317	836	-11.5	-7.8
Group II (18-26)			2	6	196	1,360	806	-14.3	-11.2
(1-26)			2	7	192	1,332	826	-12.5	-8.9

TABLE VI.

Showing the oxygen consumption, heat output, and deviation of basal metabolism from the standards.
European subjects.

Month.	Subject number.	Age.	EXPERIMENTAL.		Lowest oxygen consumption per minute in c.c.	HEAT PRODUCTION PER 24 HOURS.		DEVIATION FROM STANDARD.	
			Days.	Periods.		Total calories.	Per sq. m. calories.	Aub-duBois.	Harris-Benedict.
December 1937 ..	1	27	2	6	233	1,619	890	-6.0	-3.6
December 1937 and January 1938.	2	39	3	9	198	1,376	740	-20.2	-17.0
February 1938 ..	3	24	2	6	236	1,640	927	-2.3	-1.2
" ..	4	24	3	9	229	1,591	936	-1.3	-1.2
" ..	5	26	2	6	210	1,459	802	-15.3	-14.4
" ..	6	23	2	6	238	1,654	1,021	+7.9	+8.9
" ..	7	27	2	6	224	1,556	910	-3.9	-3.8
" ..	8	24	3	9	249	1,730	1,018	+7.4	+8.9
" ..	9	23	3	9	226	1,570	924	-2.6	-2.2
" ..	10	23	3	9	240	1,668	970	+2.4	+2.5
" ..	11	24	2	6	215	1,494	782	-17.4	-15.6
" ..	12	22	3	9	199	1,383	814	-14.1	-14.8
" ..	13	23	2	6	218	1,515	935	-1.5	-0.1
" ..	14	21	2	6	218	1,515	953	+0.3	+1.3
February and March 1938.	15	22	2	6	227	1,577	950	+0.3	-0.3
March 1938 ..	16	25	2	6	233	1,619	915	-3.5	-3.2
" ..	17	23	3	9	198	1,376	839	-11.7	-11.4
" ..	18	22	2	6	246	1,709	924	-2.4	-3.7
" ..	19	24	2	6	248	1,723	973	+2.4	+2.9
" ..	20	27	2	6	206	1,431	911	-3.8	-1.8
Average	25	2	7	224	1,560	907	-4.3	-3.5

TABLE VII.
Showing the frequency distribution of basal metabolism in the subjects studied.

INDIAN SUBJECTS.				EUROPEAN SUBJECTS.			
AUB-DUBOIS STANDARD.		HARRIS-BENEDICT STANDARD.		AUB-DUBOIS STANDARD.		HARRIS-BENEDICT STANDARD.	
Range, per cent.	Frequency.	Frequency.	Frequency.	Range, per cent.	Frequency.	Frequency.	Frequency.
-1.0 to -2.0	0	2	0	+8.0 to +10.0	0	3	3
-2.1 to -3.0	0	1	0	+6.1 to +8.0	1	0	0
-3.1 to -4.0	0	0	0	+4.1 to +6.0	0	0	0
-4.1 to -5.0	0	4	4	+2.1 to +4.0	3	2	2
-5.1 to -6.0	0	1	1	0 to +2.0	2	1	1
-6.1 to -7.0	4	1	1	-2.0 to 0	2	3	3
-7.1 to -8.0	1	2	2	-4.0 to -2.1	6	0	0
-8.1 to -9.0	1	1	1	-6.0 to -4.1	1	0	0
-9.1 to -10.0	0	1	1	-8.0 to -6.1	0	0	0
-10.1 to -11.0	3	4	4	-10.0 to -8.1	0	1	1
-11.1 to -12.0	4	1	1	-12.0 to -10.1	0	0	0
-12.1 to -13.0	2	4	4	-14.0 to -12.1	2	3	3
-13.1 to -14.0	1	0	0	-16.0 to -14.1	1	1	1
-14.1 to -15.0	1	3	3	-18.0 to -16.1	0
-15.1 to -16.0	3	0	0	-20.0 to -18.1	0
-16.1 to -17.0	1	0	0	-22.0 to -20.1	1
-17.1 to -18.0	1	0	0				
-18.1 to -19.0	2	0	0				
-19.1 to -20.0	0	0	0				
-20.0 to -21.0	0	0	0				
Mean basal metabolism	..	-3.9 per cent.	..	Mean basal metabolism	..	-3.4 per cent.	..
Standard deviation	..	4.2	..	Standard deviation	..	7.4	..
Co-efficient of variation	..	47 per cent.	..				The scatter is nearly twice the mean.

energy was 836 calories a day. This is far below the average 907 calories a day per square metre for the European. Europeans varied in their heat output for 24 hours from 1,376 calories to 1,723 calories, with an average of 1,560 calories. This, on per square metre basis, amounts to 740 to 1,021 calories a day.

If roughly twice the basal expenditure of energy be taken to represent the heat output per day on moderate work, the average for all the Indians investigated works out to $1,332 \times 2 = 2,664$, in round figures, roughly, 2,700 calories. Aykroyd and Krishnan (1937) in a paper on diet surveys in South India calculated the average calorific value of food consumed in 29 families consisting of 168 persons, to be 2,400 per day per consumption unit. When one remembers the diet surveys were made on working-class people, it is probable that the calorific intake was inadequate.

BASAL METABOLISM.

Table VII gives the frequency distribution of the basal metabolism of subjects studied in this investigation according to Aub-duBois and Harris-Benedict prediction standards.

The difference in basal metabolism between the two races (Indians and Europeans) is statistically significant* both by Aub-duBois and Harris-Benedict standards.

Difference in basal metabolism in Indians between those who are resident in hills over three years (Group I, 1-17) and those whose stay on the hills is only two months (Group II, 17-26).

	GROUP I.		GROUP II.	
	Aub-duBois.	Harris-Benedict.	Aub-duBois.	Harris-Benedict.
Mean basal metabolism ..	-11.5 per cent	-7.6 per cent	-14.2 per cent	-11.2 per cent.
Standard deviation ..	4.1	4.1	2.5	3.14
Co-efficient of variation ..	35 per cent	54 per cent	18 per cent	28 per cent.

The difference in basal metabolism in the two groups is significant using Harris-Benedict standard but not with Aub-duBois.

Table VIII gives a comparative statement of basal metabolism investigated by a few workers recently in the tropics or sub-tropics, both on the indigenous people and the Europeans with the results of the present investigation.

* The formula $\sqrt{\frac{\sigma^2}{n_1} + \frac{\sigma^2}{n_2}}$ is used to determine significance of difference in the means of two groups. σ = standard deviation; n_1 and n_2 number of subjects in the two groups.

TABLE VIII.
A comparative statement of observation on basal metabolism.

Year.	Name of worker and place of observation.	Race.	Duration of residence in the tropics.	Number of subjects.	DEVIATION STANDARD FROM	
					Aub-duBois, per cent.	Harris-Benedict, per cent.
1931	Radsma (Batavia)	European men	3 months 1 year 2 years or more	8 7 26	-10.8 -4.0 -2.9	-10.6 -4.3 -3.0
1931 1934	Mason and Benedict (Madras). Mason (Madras)	South Indian women European women in Madras.	Varied .. from 24 months to 31 years.	54 34	-17.2 -12.5	-16.9 -7.9
1937	Miller (Hawaii)	Caucasian men Chinese males	Varying periods {	25 25	-8.1 -6.4	-6.7 -5.3
1937	Kilborn and Benedict (China)	Chinese males " females Caucasian females " males Miao Chinese males	54 14 21 5 23	-2.4 -3.3 -3.1 -5.8 +9.2	+1.9 -3.7 +1.8 -0.3 +16.8
1937	Oliveiro (Singapore)	Seven races represented.	..	28	-4.8	..
1932	Krishnan and Vareed (Madras)	Indian men	..	54	-12.0	..
1938	Coonoor and Wellington	Indian men " Europeans	Over 3 years " 2 months 18 for 5 months 1 for 24 " and 1 for 3 years	Group I (17) Group II (9) All Indian (26)	-11.5 -14.2 -12.4 -4.3	-7.6 -11.2 -8.9 -3.4

DISCUSSION ON BASAL METABOLISM.

The literature dealing with the effect of tropical climate on basal metabolism both on the tropical races and Europeans is so extensive that it is not possible to review them here. Reference will be made to a few of the more recent works. Table I summarized previous work on Indian subjects. Determinations in other tropical countries carried out by Radsma (1931) in Batavia, Miller (1937) in Hawaii, Kilborn and Benedict (1937) in China, Oliveira (1937) in Malaya have all pointed out how race and tropical climate influence basal metabolism. Radsma (*loc. cit.*) and Mason (*loc. cit.*) find a lowering of the basal metabolism of the European in the tropics. Miller (*loc. cit.*) finds a fall in metabolism in European people in Hawaii but it does not differ from the Chinese in Hawaii. Kilborn and Benedict (*loc. cit.*) find an increase in two Chinese races. Oliveira (*loc. cit.*) in Singapore concludes there is no difference in basal metabolism among his subjects drawn from seven races.

Among Indians in the present investigation an average basal metabolism of — 12·4 per cent Aub-duBois with a standard deviation of 3·8 and a co-efficient of variation of 30·6 per cent and — 8·9 per cent Harris-Benedict standard, with a standard deviation of 4·2 and co-efficient of variation of 47 per cent, have been observed. These values are in close agreement with Krishnan and Vareed's (1932) in Madras. In the two groups in Indians, group I showed a basal metabolic level of — 11·5 per cent Aub-duBois and — 7·6 per cent Harris-Benedict standards, with standard deviation of 4·1 and co-efficient of variation of 35 per cent and 54 per cent respectively, whereas group II showed an average of — 14·2 per cent Aub-duBois and — 11·2 per cent Harris-Benedict with standard deviations of 2·5 and 3·1 and co-efficients of variation of 17·6 per cent and 28·2 per cent. The scatter in the basal metabolism level in the second group is smaller than in the first. The difference of 2·7 per cent on Aub-duBois standard is not found to be significant, but on the Harris-Benedict standard the difference amounts to 3·6 per cent which is statistically significant, the first group showing a higher result. This difference could be partly explained as due to the difference in the average age, the average for the first group being 30 years and for the second 33 years. It is possible also that the longer stay on the hills of the first group would have benefited them, and put up the metabolic level at a slightly higher rate. The effect of altitude on basal metabolism is to be more widely studied. Similarly, among the Europeans studied the average basal metabolism was — 4·3 per cent Aub-duBois and — 3·4 per cent Harris-Benedict standards with a standard deviation of 7·4. The variation in basal metabolism is very great here, from + 7·9 per cent to — 20·2 per cent Aub-duBois standard. The average — 4·3 per cent must be considered normal, for even in Europe and America the Aub-duBois prediction values are said to be 4 per cent too high. But one cannot ignore low values such as — 20·2 per cent in subject No. 2, — 17·4 per cent and — 15·3 per cent in subjects Nos. 11 and 5, — 14·1 per cent in subject No. 12 and — 11·7 per cent in subject No. 17. The cause of this very low metabolism must remain for the present unexplained. The remaining 15 are all within the normal limits for Europeans. The average heat production of soldiers in England with an average surface area of 1·70 square metre was found by Cathcart and Orr (1919, quoted by Orr, 1938) to be 1,542 calories a day under basal conditions. In the Wellington subjects the average surface area was 1·73 square metres and the average heat expenditure

per day was calculated to be 1,560 calories (basal). This again tends to show that there is no appreciable change in the metabolism of the group of Europeans investigated in Coonoor and Wellington to that regarded as normal in England.

If the view that the Europeans in the tropics have a lowered metabolism be upheld, presumably the hills with a cool and bracing dry climate tend to raise the metabolism; the slightly raised metabolism of the Indian subjects 1-17 who are resident on the hills for over three years is found to be significantly higher over the new-comers to the hills. Even then there is a wide difference between the average basal metabolism of the Europeans and Indians on the hills in the present study, so that the view that race *per se* as an influencing factor over basal metabolism cannot be denied.

SUMMARY.

1. The basal metabolism of 26 Indian and 20 European men has been determined under identical climatic conditions in Coonoor at an altitude of 6,000 feet above sea-level.

2. The Indian on the average has his basal metabolism 8 per cent below the European. It is very likely this difference is due to a racial factor.

ACKNOWLEDGMENTS.

The author wishes to thank Dr. E. D. Mason of Women's Christian College, Madras, for much expert advice and Colonel K. P. Mackenzie and the staff of the British Military Hospital who provided facilities to carry out the tests on the European medical orderlies. It is a great pleasure to acknowledge the author's indebtedness to them, and also the laboratory workers, assistants, and the orderlies in the British Military Hospital, who volunteered as subjects for this investigation.

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A CHEMICAL METHOD FOR THE ESTIMATION OF NICOTINIC ACID IN BIOLOGICAL MATERIALS.

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INTRODUCTION.

It now appears to be well established that pellagra in human beings (Fouts, Helmer, Lepkovsky and Jukes, 1937 ; Smith, Ruffin and Smith, 1937 ; Spies, Cooper and Blankenhorn, 1938 ; Spies, 1938), black-tongue in dogs (Elvehjem *et al.*, 1937 ; Dann, 1937 ; Street and Cowgill, 1937) and pellagra-like symptoms in pigs produced by feeding on a Goldberger pellagra-producing diet (Chick *et al.*, 1938) can be cured by the administration of nicotinic acid and that nicotinic acid is probably identical with or a precursor of the pellagra-preventive (p-p) factor.

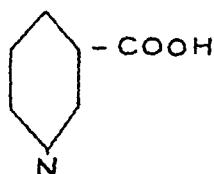
Mainly through the careful investigations of Goldberger and his collaborators, data regarding the comparative value of certain foodstuffs in the treatment or prevention of pellagra are available (Sebrell, 1934); but there are no figures for the amount of nicotinic acid in foods. Hence the question of estimating the nicotinic-acid content of foodstuffs is of immediate importance and interest.

This paper describes a new chemical method of determining nicotinic acid in foods. A preliminary account has already appeared elsewhere (Swaminathan, 1938).

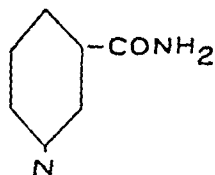
PRINCIPLE.

Nicotinic acid (1) contains a pyridine nucleus, and the method depends on the reaction described for pyridine by previous investigators (Tallantyre, 1930; Kulikow and Krestowosdwigenskaja, 1930; Strafford and Pary-Jones, 1933), in which the pyridine nucleus is broken down by cyanogen bromide and aniline to give a compound which is yellow in colour and can be estimated colorimetrically. The coloured complex so produced is a derivative of glutonaldehyde $R.NH.CH : CH.CH : CH.CH.NR.HBR$.

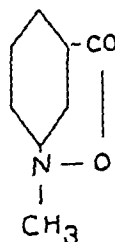
The colour is probably given by all pyridine compounds. Of these only nicotinic acid (I) and its amide (II) are biologically active as far as we know (Elvehjem *et al.*, 1938). The amide can be readily converted into the acid by boiling with dilute mineral acids and estimated as nicotinic acid.



Nicotinic acid
I



Nicotinamide
II



Trigonellin
III

Other pyridine bases present in foodstuffs include the betain trigonellin (III). This substance is probably not biologically active, as it is found in considerable amounts in peas and fenugreek seeds (Barger, 1914), which are ingredients of a pellagra-producing diet (Chick *et al.*, *loc. cit.*; Ellinger *et al.*, 1937; *B. M. J.*, 1938). Further, recently it has been shown by Woolley, Strong and Madden (1938) that trigonellin possesses no curative activity for black-tongue in dogs. A specimen of trigonellin tested gave at most 20 per cent of the colour given by nicotinic acid; whether this is due to any contamination of the sample by nicotinic acid is being investigated. It also appears from the low values obtained for the nicotinic-acid content of peas and fenugreek seeds that trigonellin probably does not interfere in the estimation of nicotinic acid by the procedure described below. Pyridine and its homologues may occur in traces. These can be readily removed by steam distillation in an alkaline medium.

METHOD.

The method consists in preparing aqueous extracts of foodstuffs suitable for colorimetric estimation and applying the procedure outlined below. Pyridine and its homologues may be determined separately in aliquots and the corresponding values subtracted from the total nicotinic-acid (nicotinic acid + pyridine bases) content to obtain the true nicotinic-acid content.

Solutions required :—

- (1) Standard nicotinic acid (strong) 1 c.c. = 1 mg. nicotinic acid.
- (2) Standard nicotinic acid (dilute) 1 c.c. = 10 μ g. nicotinic acid.
- (3) Aqueous solution of cyanogen bromide: this is prepared fresh by decolorizing in the cold a saturated solution of bromine in water by the gradual addition of 10 per cent sodium cyanide (A. R.).
- (4) Saturated aqueous solution of aniline: this is also freshly prepared by dissolving aniline in distilled water.
- (5) Sulphuric acid N/10 (approx.).
- (6) Sodium hydroxide N/10 (approx.).

Procedure for the preparation of aqueous extracts of foodstuffs suitable for colorimetric estimation.

A weighed quantity of the finely ground or minced material is placed in distilled water. The mixture is vigorously stirred and brought to the boil. (In the case of substances rich in starch it is heated on the water-bath for 20 minutes.) It is then allowed to cool and centrifuged. The clear supernatant is poured off into a clean beaker and the residue extracted twice in the above manner. To the combined extract, solid lead acetate* is added to precipitate proteins and their derivatives. The mixture is warmed to about 60°C. when the precipitate is well flocculated and part of the colouring matter is removed. It is then allowed to cool and centrifuged. By careful decantation the supernatant is separated and the residue is washed by stirring up with a little hot water, centrifuged, and the washing added to the main bulk of the extract. A slight excess of sulphuric acid (N/10 approx.) is added to the extract to precipitate the excess of lead present. The lead sulphate is removed by centrifuging and the clear supernatant is carefully separated. The lead sulphate precipitate is washed, by stirring up with a little hot water and the washings added to the main bulk of the extract. Most of the colour is removed at this stage. The extract is brought to pH 6.0, evaporated to a small bulk, and acidified by the addition of concentrated hydrochloric acid to make about 5 per cent and boiled for half an hour to decompose any nicotinamide present. It is then allowed to cool, brought to pH 10.0 by the addition of sodium hydroxide (N/10 approx.), and any colour present removed by boiling with charcoal. The charcoal is washed by boiling with a little water adjusted to pH 10.0 and the washings added to the main bulk. The clear, colourless extract is adjusted to pH 7.0, concentrated on the water-bath to a small bulk, if necessary, and made to a convenient volume. Aliquots were used for the estimation of nicotinic acid and pyridine and its homologues.

Estimation of pyridine and its homologues.—An aliquot of the extract is made strongly alkaline by the addition of sodium hydroxide (N/10 approx.) and steam-distilled till about 150 c.c. to 200 c.c. of the distillate is collected in a receiver containing a little dilute sulphuric acid, the end of the condenser just dipping in the acid. It is finally brought to pH 7.0 and made up to volume. Aliquots were used for the estimation of pyridine and its homologues, against nicotinic acid standard. This value is subtracted from the total nicotinic acid (nicotinic acid + pyridine bases) obtained from the original extract to obtain the true nicotinic acid present.

Procedure for colorimetric comparison.—In the previous communication (Swaminathan, *loc. cit.*) a procedure was described for the colorimetric estimation of nicotinic acid present in the extracts obtained from foodstuffs. It was found from experience that great care was necessary in purifying the amyl alcohol. The yellow colour obtained with nicotinic acid was found to fade when extracted by amyl alcohol purified according to the method described by Strafford and Parry-Jones (*loc. cit.*), whereas the colour obtained with pyridine was quite

* The method described in the text for removal of interfering substances has been extensively tested and found to be satisfactory. Recently, simpler methods have been tried out, and with a few foods comparable results have been obtained by omitting the lead precipitation, and boiling with 2 per cent hydrochloric acid for 30 minutes instead.

stable. The difficulty was overcome by using amyl alcohol purified as follows: Commercial amyl alcohol is extracted twice with 10 per cent sulphuric acid, washed with water, extracted twice with 10 per cent sodium hydroxide, washed free from alkali with distilled water, dried over anhydrous sodium sulphate, and distilled. Further the procedure was time consuming. A simpler procedure is described below (which avoids the use of amyl alcohol), by using which reliable results are obtained in a short time:—

Aliquots of the extract and the standard nicotinic acid (dilute) (20 μ g. to 50 μ g.) are measured into 25 c.c. graduated flasks and diluted to the same volume 16 c.c. This must be done accurately to within 0.5 c.c. Two drops of freshly prepared saturated aqueous aniline solution (v. i.) and 4 c.c. of freshly prepared aqueous cyanogen bromide are then added to each, the mixtures shaken, and allowed to stand. A bright yellow colour develops and reaches its maximum intensity in two minutes. Four c.c. of saturated aqueous aniline solution are then added and the whole made up accurately to 25 c.c. with distilled water. The colours are compared in a colorimeter immediately. The colours are quite stable for about half an hour after which they begin to fade.

EXPERIMENTAL.

Proportionality of colour.—Measured quantities of standard nicotinic-acid solution (dilute) corresponding to amounts from 10 μ g. to 50 μ g. nicotinic acid were treated with cyanogen bromide and aniline as described above. The coloured solution containing 30 μ g. was used as the standard against which the other solutions were read. The results were as follows:—

Taken	.. 10	20	30	40	50
Found	.. 10.5	21	30	39	51

(Figures represent μ g. nicotinic acid.)

Recovery of nicotinic acid added to foodstuffs in the form of nicotinic acid and nicotinamide.—Different amounts of nicotinic acid and nicotinamide were added to known amounts of yeast and raw milled rice and the recovery was good in all the cases. The results are shown in Table I:—

TABLE I.

Foodstuff + nicotinic acid + nicotinamide.	Total nicotinic acid found, mg.	Recovery, per cent.
Raw milled rice, 20 g.	0.40	..
Raw milled rice, 20 g. + 0.5 mg. nicotinic acid + 0.5 mg. nicotinamide.	1.39	99
Raw milled rice, 20 g. + 0.3 mg. nicotinic acid + 0.3 mg. nicotinamide.	0.97	97
Dried yeast, 10 g.	5.71	..
Dried yeast, 10 g. + 1 mg. nicotinic acid + 1 mg. nicotinamide	7.77	103
Dried yeast, 10 g. + 5 mg. nicotinic acid + 5 mg. nicotinamide	15.87	102

Removal of pyridine added to an extract of yeast.

To an extract prepared from yeast (10 g.) in the above manner, 1 mg. of pyridine was added and the whole made up to volume. Aliquots were used for the estimation of total nicotinic acid (nicotinic acid + pyridine) against nicotinic-acid standard, and also for the estimation of pyridine in the steam distillate. The value obtained by difference for the nicotinic-acid content of yeast was within the experimental error.

			Nicotinic acid, mg.
Yeast, 10 g. + 1 mg. pyridine	..		8.99
Pyridine, 1 mg. in the steam distillate	..		3.33
Yeast, 10 g. (obtained by difference)	..		5.66
Yeast, 10 g. (obtained directly)	..		5.71
Error	1 per cent	

ANALYSIS OF FOODSTUFFS.

The above method was applied to a few foodstuffs.

TABLE II.

Name of foodstuff.	Nicotinic acid, mg. per cent.	Pyridine bases.
(a) Yeast, dried (brewers'), I ..	62.50	..
(b) Yeast, dried (brewers'), II ..	57.14	Nil
(b) Peanut meal (ether extracted)	16.66	..
(b) Liver, sheep (fresh) ..	12.50	Nil
(a) Skimmed milk powder ..	10.53	..
(a) Wheat, whole ..	5.33	..
(a) Red gram (<i>Cajanus indicus</i>) ..	5.33	..
(a) Soya bean ..	4.85	..

(a) By the amyl alcohol procedure described in a previous paper (Swaminathan, *loc. cit.*).

(b) By the procedure described in this paper.

TABLE II—concl'd.

Name of foodstuffs.	Nicotinic acid, mg. per cent.	Pyridine bases.
(a) Bengal gram	4.74	..
(a) Ragi (<i>Eleusina coracana</i>)	3.08	..
(a) Cambu (<i>Pennisetum typhoideum</i>)	2.50	..
(a) Rice, parboiled, home-pounded	2.78	..
(a) Rice, raw milled, I	2.38	..
(b) Rice, raw milled, II	2.00	Nil
(a) Cholam (<i>Sorghum vulgare</i>)	1.82	..
(b) Pepper, green (<i>Piper nigrum</i>)	1.17	..
(b) Fenugreek seeds	1.03	Nil
(b) Peas, dry	1.00	Nil
(a) Maize, dry (white), I	1.48	..
(b) Maize, dry (white), II	0.66	Nil

(a) By the amyl alcohol procedure described in a previous paper
(Swaminathan, *loc. cit.*).

(b) By the procedure described in this paper.

Estimation of nicotinic acid in blood.

A knowledge of the level of nicotinic acid in normal blood should be of importance in the diagnosis of nicotinic-acid deficiency. The method can be adopted for estimations in blood, by precipitating the proteins using tungstic acid (Folin and Wu, 1919) and applying the colorimetric procedure.

METHOD.

To 35 c.c. of water (7 volumes) in a 80 c.c. centrifuge tube, 5 c.c. of freshly drawn blood (1 volume) are added and mixed by gentle agitation. Five c.c. of sodium tungstate 10 per cent (1 volume) are then added followed by 5 c.c. of $\frac{2}{3}$ N sulphuric acid (1 volume). The tube is first gently shaken to mix the contents during the addition of the reagents and finally vigorously shaken by inserting a rubber stopper. It is then centrifuged and the clear supernatant is carefully separated and measured (10 c.c. correspond to 1 c.c. of blood). Enough concentrated hydrochloric acid is then added to make a concentration of 5 per cent and the whole brought to the boil to decompose any nicotinamide present. It is then allowed to cool, brought to pH 7.0 by the addition of sodium hydroxide (10/N approx.) and concentrated to a small bulk (10 c.c. to 12 c.c.)

on a water-bath and used for the colorimetric estimation of the nicotinic acid present. The method was applied to three samples of human blood and in one case the recovery of added nicotinic acid was good. The results are given below:—

Blood sample.	Mg. nicotinic acid per 100 c.c. blood.
1	0.53
2	0.37
3	0.33

Recovery of added nicotinic acid.	Nicotinic acid found, μ g.
Blood, 4 c.c.	14
Blood, 4 c.c. + 20 μ g. nicotinic acid	36
Recovery of added nicotinic acid ..	110 per cent

DISCUSSION.

From the above results, it appears that the chemical method for estimating nicotinic acid in foodstuffs is suitable for application to a wide range of materials containing varying amounts of nicotinic acid (0.66 mg. to 57.14 mg. per cent). The recovery of added nicotinic acid is good.

Fenugreek seeds and peas have been shown to contain fair amounts of trigonellin the methylbetain of nicotinic acid (Jahns, 1885; Schulz and Winterstein, 1910). From the low values obtained for these foodstuffs, it appears that trigonellin probably does not react with the reagents to give an yellow colour or takes a longer time than nicotinic acid to give the colour. This point is being further investigated. There was no trace of pyridine and its homologues in the few foodstuffs examined.

The low value (0.66 mg.) for maize obtained in the present investigation is particularly interesting because of its association with pellagra. On the other hand dried brewers' yeast, which is known to be a rich source of the 'pellagra-preventive (p-p) vitamin', contains 57.14 mg. Vickery (1926) isolated nicotinic acid from dried brewers' yeast in an yield of 0.018 per cent. Raw milled rice contains about three times as much nicotinic acid as maize. While true pellagra is rare in India, a pellagra-like condition (stomatitis) is extremely common and it is quite possible that deficiency of nicotinic acid is an important defect of Indian diets based on rice and millet.

A systematic examination of the nicotinic-acid content of most of the common Indian foods is in progress.

SUMMARY AND CONCLUSION.

1. A chemical method for the estimation of nicotinic acid (pellagra-preventive vitamin) in foodstuffs has been described. The method is based on the yellow colour given by the pyridine ring when acted upon by cyanogen bromide and aniline.
2. It takes only two minutes for the maximum development of the colour in aqueous solution and the colour is stable for about half an hour which greatly facilitates the colorimetric measurements.
3. The colour reaction is extremely sensitive, 0.01 mg. of nicotinic acid being easily estimated and the colour developed is strictly proportional to the concentration of nicotinic acid.
4. A low value of 0.66 mg. per cent was obtained for maize which has been associated with pellagra.
5. It appears from the low values obtained for peas and fenugreek seeds that trigonellin, the methylbetain of nicotinic acid, does not probably interfere in the estimation of nicotinic acid under the experimental conditions.

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*Note (added in proof).—*A pure specimen of trigonellin, recently obtained from Dr. B. C. J. G. Knight, gave no colour at all with the cyanogen-bromide and aniline reagents.—M. S.

THE VITAMIN-A ACTIVITY OF SOME FISH OILS AND VEGETABLE FOODS.

PARTS I and II.

BY

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Part I.

THE VITAMIN-A CONTENT OF SOME FISH OILS.

A FEW fish oils were analysed spectrographically for their vitamin-A content and the results published in previous communications (De, 1935*a*, 1937). The assay of vitamin A of the various Indian foodstuffs is being systematically carried out in these laboratories, and forms a part of the 'food survey work' in progress at Coonoor. Considerable data on the nutritive value of Indian foodstuffs have already been worked out and reported, in Health Bulletin No. 23 (1937) published by the Government of India. Part I of the present paper records further data on the spectrographic assay of the vitamin-A content of an additional number of 30 fish oils. These samples of oils were forwarded to these laboratories for vitamin-A assay by the Fisheries Department of the Government of Madras. The method of assay adopted was the same as that described previously by one of us (De, 1935*b*, 1937).

Most of the fish oils examined were prepared at the Madras and Tanur Stations of the Madras Government Fisheries Department. The fishes were caught from the sea round the Madras Presidency coasts and in the river at the Metur Dam. The method of extracting the oils, as reported by the Fisheries Department, was as follows: 'The organs washed free from blood were cooked in a sufficient quantity of water in a heavily tinned copper vessel. The oil rising to the top was skimmed

off with a wooden ladle into an enamel vessel. The oil was then clarified by repeated washing with lukewarm water and filtered through paper'.

The data of the vitamin-A assay work are set out in Table I. Two samples of halibut-liver oil preparations by Parke, Davis & Co. and Crookes Laboratories respectively were also analysed. Table I is set out in descending order of vitamin-A activity.

TABLE I.

Material.	Vitamin-A content per gramme in micrograms.
Halibut-liver oil (Parke, Davis & Co.) (55,000 I. U. per g.) ..	21,500 equal to 55,000 I.U. of vitamin A.
Halibut-liver oil (Crookes) (52,000 I. U. per g.)	20,400 equal to 53,000 I.U. of vitamin A.
Shark-liver oil (hammer-headed) (<i>Zygaena Blochi</i>)	7,600
„ „ (man-eating species)	4,200
„ „	2,400
Sawfish ' Vela ' (liver oil)	2,250
Shark-liver oil (from Travancore)	1,750
Sawfish-liver oil (<i>Pristis Perrotteti</i>)	1,125
Indian cod-liver oil (from Calicut)	1,000
Eagle-ray-liver oil (<i>Aetobatis guttata</i> , Shaw)	440
Norwegian cod-liver oil (6 samples)	560 to 156 : average = 320.
Nethal (liver oil)	310
Soman thirukkai (liver oil)	290
<i>Arius</i> sp. (liver oil)	250
<i>Barbus dubius</i> (whole body)	190
Manthal fish (liver oil)	190
Karakutti (liver oil)	135
Cat fish liver oil	44
<i>Catla Catla</i> (whole body)	19
Turtle oil (whole body)	5
Sardine (whole body), classes I and II	1 to 2
Kuttippu (<i>Lactarius Lactaries</i>) (whole body)	1

TABLE II—concl'd.

Name.	Botanical name.	Source.	Carotene content in micrograms per gramme.
nut-meg	<i>Myristica fragrans</i>	Local	Trace.
„ rind	„	„	0.08
lmyra fruit, tender, juice only.	<i>Borassus flabellifer</i>	Tinnevely	20.00
ni-varagu	<i>Panicum miliaceum</i>	Coimbatore	Trace.
pads	Local	Nil
ion fruit	<i>Passiflora edulis</i>	„	0.90
ber, green	<i>Piper nigrum</i>	„	6.80
mmon, ripe	<i>Diospyros kaka</i>	„	17.00
chio nut	<i>Pistacia vera</i>	„	2.40
uin, red variety ..	<i>Musa paradisiaca</i>	„	3.50
in, stem	„	„	Nil
.. ..	<i>Vitis vinifera</i>	„	Trace.
or leaves	<i>Carthamus tinctorius</i>	„	55.00
millet	<i>Panicum crusgalli</i> var. <i>frumantaceum</i> .	Coimbatore	Trace.
t	<i>Anona squamosa</i>	„	Trace.
ne, juice only ..	<i>Saccharum officinarum</i>	Local	0.10
ans	<i>Canavalia ensiformis</i>	„	0.40
our	<i>Borassus flabellifer</i>	Poona	Nil
.. ..	<i>Manihot utilisima</i>	Travancore	Nil
nut)	<i>Mucifera</i>	Local	Nil
.. ..	<i>Andromeda</i>	„	5.40
.. ..	<i>regia</i>	„	10.00
.. ..	<i>va</i>	„	Nil
..	Setti brewery	1.10

, approximately one I. U. of vitamin

vegetables and certain ripe fruits
y poor sources.

off with a wooden ladle into an enamel vessel. The oil was then clarified by repeated washing with lukewarm water and filtered through paper'.

The data of the vitamin-A assay work are set out in Table I. Two samples of halibut-liver oil preparations by Parke, Davis & Co. and Crookes Laboratories respectively were also analysed. Table I is set out in descending order of vitamin-A activity.

TABLE I.

Material.	Vitamin-A content per gramme in micrograms.
Halibut-liver oil (Parke, Davis & Co.) (55,000 I. U. per g.) ..	21,500 equal to 55,900 I.U. of vitamin A.
Halibut-liver oil (Crookes) (52,000 I. U. per g.)	20,400 equal to 53,000 I.U. of vitamin A.
Shark-liver oil (hammer-headed) (<i>Zygaena Blochi</i>)	7,600
" " (man-eating species)	4,200
" "	2,400
Sawfish ' Vela ' (liver oil)	2,250
Shark-liver oil (from Travancore)	1,750
Sawfish-liver oil (<i>Pristis Perrotteti</i>)	1,125
Indian cod-liver oil (from Calicut)	1,000
Eagle-ray-liver oil (<i>Ætobatis guttata</i> , Shaw)	440
Norwegian cod-liver oil (6 samples)	560 to 156 : average = 320.
Nethal (liver oil)	310
Soman thirukkai (liver oil)	290
<i>Arius</i> sp. (liver oil)	250
<i>Barbus dubius</i> (whole body)	190
Manthal fish (liver oil)	190
Karakutti (liver oil)	135
Cat fish liver oil	44
<i>Catla Catla</i> (whole body)	19
Turtle oil (whole body)	5
Sardine (whole body), classes I and II	1 to 2
Kuttiippu (<i>Lactarius Lactaries</i>) (whole body)	1

TABLE II—concl'd.

Name.	Botanical name.	Source.	Carotene content in micrograms per gramme.
Nut-meg	<i>Myristica fragrans</i>	Local	Trace.
„ rind	„	„	0.08
Palmyra fruit, tender, juice only.	<i>Borassus flabellifer</i>	Tinnevely	20.00
Pani-varagu	<i>Panicum miliaceum</i>	Coimbatore	Trace.
Pappads	„	Local	Nil
Passion fruit	<i>Passiflora edulis</i>	„	0.90
Pepper, green	<i>Piper nigrum</i>	„	6.80
Persimmon, ripe	<i>Diospyros kaka</i>	„	17.00
Pistachio nut	<i>Pistacia vera</i>	„	2.40
Plantain, red variety	<i>Musa paradisiaca</i>	„	3.50
Plantain, stem	„	„	Nil
Raisins	<i>Vitis vinifera</i>	„	Trace.
Safflower leaves	<i>Carthamus tinctorius</i>	„	55.00
Sanwa millet	<i>Panicum crusgalli</i> var. <i>frumentaceum</i> .	Coimbatore	Trace.
Sita fruit	<i>Anona squamosa</i>	„	Trace.
Sugar cane, juice only	<i>Saccharum officinarum</i>	Local	0.10
Sword beans	<i>Canavalia ensiformis</i>	„	0.40
Talipot flour	<i>Borassus flabellifer</i>	Poona	Nil
Tapioca	<i>Manihot utilissima</i>	Travancore	Nil
Toddy (coco-nut)	<i>Cocos nucifera</i>	Local	Nil
Tree tomato, ripe	<i>Cyphomandra betacea</i>	„	5.40
Walnut	<i>Juglans regia</i>	„	10.00
Wheat flour, refined	<i>Triticum vulgare</i>	„	Nil
Yeast powder, dry	„	Ketti brewery	1.10

One microgram of these carotene figures corresponds to approximately one I. U. of vitamin A (De, 1937).

From Table II it is apparent that green leafy vegetables and certain ripe fruits are rich in carotene. Other food materials are very poor sources.

Name.	Botanical name.
Nut-meg	<i>Myristica fragrans</i>
„ rind ..	„
Palmyra fruit, tender, juice only.	<i>Borassus flabellifer</i>
Pani-varagu	<i>Panicum miliaceum</i>
Pappads	„
Passion fruit	<i>Passiflora edulis</i>
Pepper, green	<i>Piper nigrum</i>
Persimmon, ripe	<i>Diospyros kaki</i>
Pistachio nut	<i>Pistacia vera</i>
Plantain, red variety ..	<i>Musa paradisiaca</i>
Plantain, stem	„
Raisins	<i>Vitis vinifera</i>
Safflower leaves	<i>Carthamus tinctorius</i>
Sanwa millet	<i>Panicum crusgalli</i> var. <i>fr. mantaceum</i> .
Sita fruit	<i>Anona squamosa</i>
Sugar cane, juice only ..	<i>Saccharum officinarum</i>
Sword beans	<i>Canavalia ensiformis</i>
Talipot flour	<i>Borassus flabellifer</i>
Tapioca	<i>Manihot utilissima</i>
Toddy (coco-nut)	<i>Cocos nucifera</i>
Tree tomato, ripe	<i>Cyphomandra beta</i>
Walnut	<i>Juglans regia</i>
Wheat flour, refined ..	<i>Triticum</i>
Yeast powder, dry	„

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orted previously (De, riments were under- on a 'poor Madrassi l milk powder and ount of work has ishnani (1937a and b) milk powder and ldren. They showed id body-weight, these eneral health of the ing relieved a certain noderma, the latter sition naturally arises; vitamin A?

lition of skimmed milk diet', would influence ource of vitamin A in l animals. It may be and Steenbock's (1934) ble to man beings,

One microgram of these carotene
A (De, 1937).

From Table II it is app
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and carotene.

SUMMARY.

1. Some 30 fish oils and 45 vegetable foodstuffs have been assayed for vitamin A and carotene contents respectively.
2. Some Indian fish-liver oils were found to be many times richer in vitamin A than the Norwegian cod-liver oils.

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THE ASSIMILATION OF CAROTENE BY RATS FROM A 'POOR MADRASSI DIET': THE INFLUENCE OF SKIMMED MILK AND CALCIUM LACTATE.

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IN continuation of the carotene metabolism work reported previously (De, 1937a; Wilson, Ahmad and Majumdar, 1936) further experiments were undertaken to study the rate of assimilation of carotene by rats fed on a 'poor Madrassi diet', unsupplemented and supplemented with skimmed milk powder and calcium lactate respectively. Recently, a considerable amount of work has been reported from these laboratories by Aykroyd and Krishnan (1937a and b) revealing the importance of supplementing skimmed milk powder and calcium lactate to the diets of South Indian school children. They showed that, besides producing considerable increase in height and body-weight, these supplements bestow numerous beneficial effects to the general health of the children. It is interesting to note that skimmed milk feeding relieved a certain number of children suffering from stomatitis and phrynoderma, the latter condition being ascribed to vitamin-A deficiency. A question naturally arises; does this supplement influence metabolism of carotene and vitamin A?

It was, therefore, thought worth while to study if an addition of skimmed milk powder and also of calcium lactate to this 'poor Madrassi diet', would influence the rate of absorption of carotene, which forms the only source of vitamin A in the diet. For convenience, rats were taken as experimental animals. It may be noted that in the light of Clausens's (1933) and Baumann and Steenbock's (1934) work the results of the rat experiment may be applicable to human beings, especially to children.

Two separate and independent observations were made in this problem. In the first experiment, 3 groups of stock rats, weighing 50 grammes to 60 grammes each, were kept for 3 weeks on a synthetic vitamin-A free diet; as a consequence, the faecal matter of these rats became completely devoid of vitamin A and carotene.

Subsequently, these 3 groups of animals were supplied for 15 days with the experimental diets namely the 'poor Madrassi diet', unsupplemented and supplemented with skimmed milk powder and calcium lactate respectively. The carotene content of the diets supplied, the actual amount of food consumed daily by the different groups of rats, and the amount of carotene excreted through faecal matter, were determined in the usual way. It was found as already reported before (De, 1937a) that the faecal matter was always devoid of vitamin A, while the urine was free from both vitamin A and carotene.

The carotene contents of the various foods and the faeces were extracted directly and estimated colorimetrically by using solutions of pure carotene as standards. The strength of the standards was estimated by using a modified spectrophotometric method (De, 1937b). The difference between the amount of carotene consumed in the food and that excreted through the faeces was taken as a measure of the amount of carotene absorbed (De, 1937a). 'Absorption' in this sense, therefore, includes vitamin A or carotene that is destroyed in the digestive tract.

The composition of the vitamin-A free synthetic diet and of the 'poor Madrassi diet' was as follows :—

Composition of the synthetic vitamin-A free diet.

	Parts.	
Starch	60	
Casein	20	
Coco-nut oil ..	10	
Yeast powder ..	5	
Salt mixture (McCollum) ..	5	containing about 50 per cent of calcium salts (lactate and phosphate).

With tap water to drink.

Each rat usually took daily 10 to 15 grammes of this diet, dry-weight.

Composition of the 'poor Madrassi diet'.

Constituents.	Proportion, oz.	Carotene content* in micrograms per gramme.
Raw polished rice	21.0	0
Red gram (<i>Cajanus indicus</i>) ..	0.7	2.2
Black gram (<i>Phaseolus mungo</i>) ..	0.7	0.6
Gingelly oil (<i>Sesamum indicum</i>) ..	0.1	0
Brinjal (<i>Solanum melongena</i>) (boiled) ..	1.0	0.05
Amaranth leaves (<i>Amaranthus gangeticus</i>) ..	0.5	25.0 to 35.0
Raw plantain (<i>Musa paradisiaca</i>) (boiled) ..	0.5	0.5
Meat	0.06	trace
Coco-nut (<i>Cocos nucifera</i>) ..	0.05	0

* De (1936).

With tap water to drink.

The above quantities correspond roughly to human adult daily intake. The calcium content* of this mixed diet was 0.028 per cent. Each rat usually took 15 to 25 grammes of this mixed diet daily, thus obtaining 4 mg. to 7 mg. of calcium.

Calculated in terms of adult human intake, this diet corresponds to about 500 micrograms of carotene, 0.2 gramme of calcium, and 2,250 calories daily.

In the 'poor Madrassi diet' amaranth is practically the only source of carotene, other constituents involving only a trace. As such, in the present experiment, they are negligible as sources of carotene.

During the course of this investigation the carotene content of amaranth varied between 25 and 35 micrograms per gramme, and the intake of carotene per rat per day varied between 6 and 7 micrograms.

The above diet known in these laboratories as the 'poor Madrassi diet' (Aykroyd and Krishnan, 1937a) is substantially the same as that described in McCarrison's (1931) 'Food' as the 'poor Hindu family diet'.

The data of the present experiment are set out in the following tables:—

RAT EXPERIMENT NO. 1.

Assimilation of carotene by rats fed on the 'poor Madrassi diet'.

TABLE I.

(The 'poor Madrassi diet', unsupplemented, fed to 12 rats.)

Days.	Carotene taken by the group in micrograms.	Carotene excreted by the group in micrograms.	Percentage of carotene absorbed from difference of columns 2 and 3.
1-3 ..	250	65	58
4-6 ..	280	85	
7-9 ..	285	85	
10-12 ..	260	70	
13-15 ..	290	55	
16-21 ..	0	165	
22-25 ..	0	40	
26-27 ..	0	3	
28-30 ..	0	0	
TOTAL ..	1,365	568	

* Health Bulletin No. 23 (1937).

TABLE II.

(The 'poor Madrassi diet' plus skimmed milk powder fed to 12 rats.)
(10.5 g. of skimmed milk powder fed to 12 rats per day.)

Days.	Carotene taken by the group in micro-grams.	Carotene excreted by the group in micro-grams.	Percentage of carotene absorbed from difference of columns 2 and 3.
1-3 ..	240	60	61
4-6 ..	275	95	
7-9 ..	295	80	
10-12 ..	260	75	
13-15 ..	295	60	
16-21 ..	0	115	
22-25 ..	0	40	
26-27 ..	0	3	
28-30 ..	0	0	
TOTAL ..	1,365	528	

TABLE III.

(The 'poor Madrassi diet' plus calcium lactate fed to 12 rats.)
(1.75 g. of calcium lactate for 12 rats per day.)

Days.	Carotene taken by the group in micro-grams.	Carotene excreted by the group in micro-grams.	Percentage of carotene absorbed from difference of columns 2 and 3
1-3 ..	255	40	60
4-6 ..	280	55	
7-9 ..	275	80	
10-12 ..	260	110	
13-15 ..	290	75	
16-21 ..	0	95	
22-25 ..	0	85	
26-27 ..	0	5	
28-30 ..	0	0	
TOTAL ..	1,360	545	..

The rats were fed on the experimental diets for the 1st to 15th day and afterwards kept on the vitamin-A free synthetic diet.

From Tables I, II, and III it appears that rats assimilated 58 to 61 per cent of carotene from the 'poor Madrassi diet', unsupplemented and supplemented. The addition of skimmed milk powder and calcium lactate did not substantially influence carotene absorption. From the records of body-weight (not shown in the paper) it appeared that the skimmed milk group showed considerable increase of body-weight as compared with the groups kept on the 'poor Madrassi diet'.

unsupplemented and supplemented with calcium lactate, the latter two groups showing similar rate of increase in body-weight. The fact that addition of calcium lactate did not show any beneficial effect can be explained as follows:—

In the first series of experiments the rats were depleted by feeding them a synthetic vitamin-A free diet for 3 weeks. This feeding included about 0.5 g. of calcium salts per rat per day. During the metabolism work the rats of group III (Table III) were given only 0.15 g. of calcium lactate per rat per day. It is because of the high doses of calcium salts fed to all the rats initially that the beneficial effect of the smaller doses, given subsequently for two weeks only, could not be apparent.

In view of this fact it was considered better to use for this investigation rats which have been kept on the Madrassi diet unsupplemented and supplemented, for a considerably longer period and to deplete them by some other means not involving the use of calcium salts.

In a second series of experiments three groups of rats (5 in each group) were kept for 10 weeks on the 'poor Madrassi diet' unsupplemented and supplemented with skimmed milk powder and calcium lactate respectively. The latter two groups showed better rate of growth than the group I, the skimmed milk showing the highest rate of growth. These animals were then depleted, i.e., faeces made free from carotene, by feeding them for 3 weeks on the same Madrassi diets minus amaranth, the other constituents of the Madrassi diet involving only a trace of carotene.

The data of the metabolism work are recorded in Table IV:—

RAT EXPERIMENT NO. 2.

The assimilation of carotene by rats fed on the 'poor Madrassi diet' unsupplemented and supplemented with skimmed milk powder and calcium lactate respectively.

TABLE VI.

Days.	Carotene taken by each group of rats in micrograms.	CAROTENE EXCRETED BY THE DIFFERENT GROUPS IN MICROGRAMS.		
		Group I.	Group II.	Group III.
1-4 ..	575	70	105	90
5-7 ..	300	45	80	95
8-10 ..	275	95	120	110
11-13 ..	275	70	65	80
14-16 ..	265	335	215	250
17-20 ..	0			
21-23 ..	0	15	25	20
24-26 ..	0	5	15	10
27-30 ..	0	0	0	0
TOTAL ..	1,690	635	625	655
Percentage of carotene absorbed.		62	63	61

Each group consisted of 5 rats. From the 1st to the 16th day each one of the rats was first fed with about 0.5 g. of amaranth mixed with a little quantity of the basal diet, that is 'poor Madrassi diet' less amaranth. When this was completely eaten sufficient quantity of the basal diet was supplied. From the 17th day onwards ingestion of carotene was stopped and the rats were fed on the basal diet alone. During the course of this experiment the carotene content of amaranth varied between 35 and 60 micrograms per gramme, the intake of carotene per rat per day was between 13 and 30 micrograms.

DISCUSSION.

From Tables I, II, III, and IV it is clear that only about 60 per cent of the carotene is absorbed by rats fed on a mixed diet like the ones used. Neither skimmed milk nor calcium lactate has any significant influence towards carotene absorption. The only observed influence of these supplements was to lead to increased rate of growth and body-weight (Aykroyd and Krishnan, 1937a and b).

The above observations refer to healthy and normal animals. The possibility, however, remains that absorption of vitamin A and carotene may be affected by various factors in the presence of pathological symptoms. There have been already numerous reports (Ralli *et al.*, 1935; Heymann, 1936a and b; and other workers) which show that absorption of both vitamin A and carotene may be considerably impaired in the absence of bile, in diabetes, hyper-thyroidism and in various infectious diseases.

SUMMARY.

1. About 60 per cent of carotene ingested in food was assimilated by rats from the 'poor Madrassi diet'.
2. Skimmed milk powder and calcium lactate, when added to this mixed diet, produced acceleration of growth, but showed no significant influence on the carotene absorption.

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STUDIES IN CALCIUM AND PHOSPHORUS METABOLISM.

Part II.

THE EFFECT OF TOXIC DOSES OF VITAMIN D ON THE ASH, Ca, AND P CONTENT OF THE BONES AND ON THE EXCRETION OF N, Ca, AND P IN THE ALBINO RATS.

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INTRODUCTION.

THE fact that the administration of excessive doses of vitamin D to rats profoundly altered the calcium and phosphorus metabolism was shown by Harris and Innes (1931). Brown and Shohl (1930) observed that the effect was more pronounced with normal diets than with Steenbock's rachitogenic diet. They also observed a shift of calcium and phosphorus from faeces to urine and a reduction in bone ash in rats receiving 10,000 minimum curative doses or over per day. By feeding irradiated ergosterol to rats Watchorn (1930*a* and *b*) observed: (1) a reduced retention of both Ca and P, (2) an increase in urinary calcium but not in urinary phosphorus, and (3) a decrease in the faecal Ca and P which was not proportional to the intake of these elements. Light, Miller and Frey (1929) reported, on the other hand, a greater elimination of phosphorus than calcium during hypervitaminosis D. Commenting on the results of Watchorn, and Brown and Shohl, Harris and Innes (*loc. cit.*) remark that an increase in the dose of vitamin D first acts by promoting the absorption of Ca and P from the intestines and possibly also the excretion of these elements into the gut. In the later stages of hypervitaminosis gut function is impaired and the faecal Ca and P represent

mostly the unabsorbed material. Nicolaysen (1937) has shown that the absorption of phosphates from the gut does not differ in the normal and vitamin D deficient rats. It does not necessarily follow, however, that since the deficiency of vitamin D did not affect the absorption of phosphates its excess also should be without any effect. Kern, Montgomery and Still (1931) observed a rise in the urinary Ca and P of the rats receiving high doses of irradiated ergosterol. They found that the animals were throughout the experimental period on positive Ca and P balance and that the urinary nitrogen was roughly proportional to the food intake.

Although it is generally agreed that hypervitaminosis D results in the demineralization of bones, no adequate data are available which deal with the respective proportions of Ca and P in the demineralized bone. It is generally assumed that both Ca and P are withdrawn from the bones in the proportion in which they exist in the bone. Kern, Montgomery and Still (*loc. cit.*) found no change in the Ca : P : CO₂ content of the bones of rats receiving excessive doses of irradiated ergosterol. Jones and Robson (1931) contend that hypervitaminosis D does not result in the demineralization of bone but that it probably interferes with the process of deposition of bone salts.

In the following pages is presented an account of the effects of toxic doses of irradiated ergosterol on the calcium, phosphorus, and nitrogen metabolism in albino rats.

EXPERIMENTAL.

Rats from different litters of known dietary history were kept separately in metabolic cages fitted for the collection of urine and faeces. The food prepared according to an earlier communication (Patwardhan and Chitre, 1938—see Table I, diet I) was given in a powdered form in special containers so that no food was lost by spilling. Food and water were given *ad lib.* A record of food intake was kept, however, from day to day by weighing a sample of food given and the food left over the next day, after drying at 100°C. Vigantol which is a Merck's preparation of irradiated ergosterol of the value of 50,000 I. U. per mg. dissolved in oil was used in these experiments. To six rats it was administered orally for varying periods at the rate of 4,500 I. U. per rat per day. To four rats an equivalent amount was administered subcutaneously. The urine and faeces were collected every two days and analysed. Calcium and phosphorus were determined by the methods described in the earlier communication. Nitrogen was estimated by the Kjeldahl's method.

The results given in Tables I and II are illustrative of the series of ten experiments carried out on similar lines. To rat 462 Vigantol was administered orally and to rat 465 an identical amount subcutaneously. The administration of the toxic doses of vitamin D and the balance experiments were carried out for 26 to 34 days. In the cases of the rats receiving Vigantol subcutaneously the experiment stopped with the death of the animals. Rat 462 and others receiving Vigantol orally were killed at the end of the experimental period. The figures for N, P, and Ca in the tables are in milligrams of these elements per day per rat for each period of two days. The experiments with other rats which have not been included here will be mentioned later on.

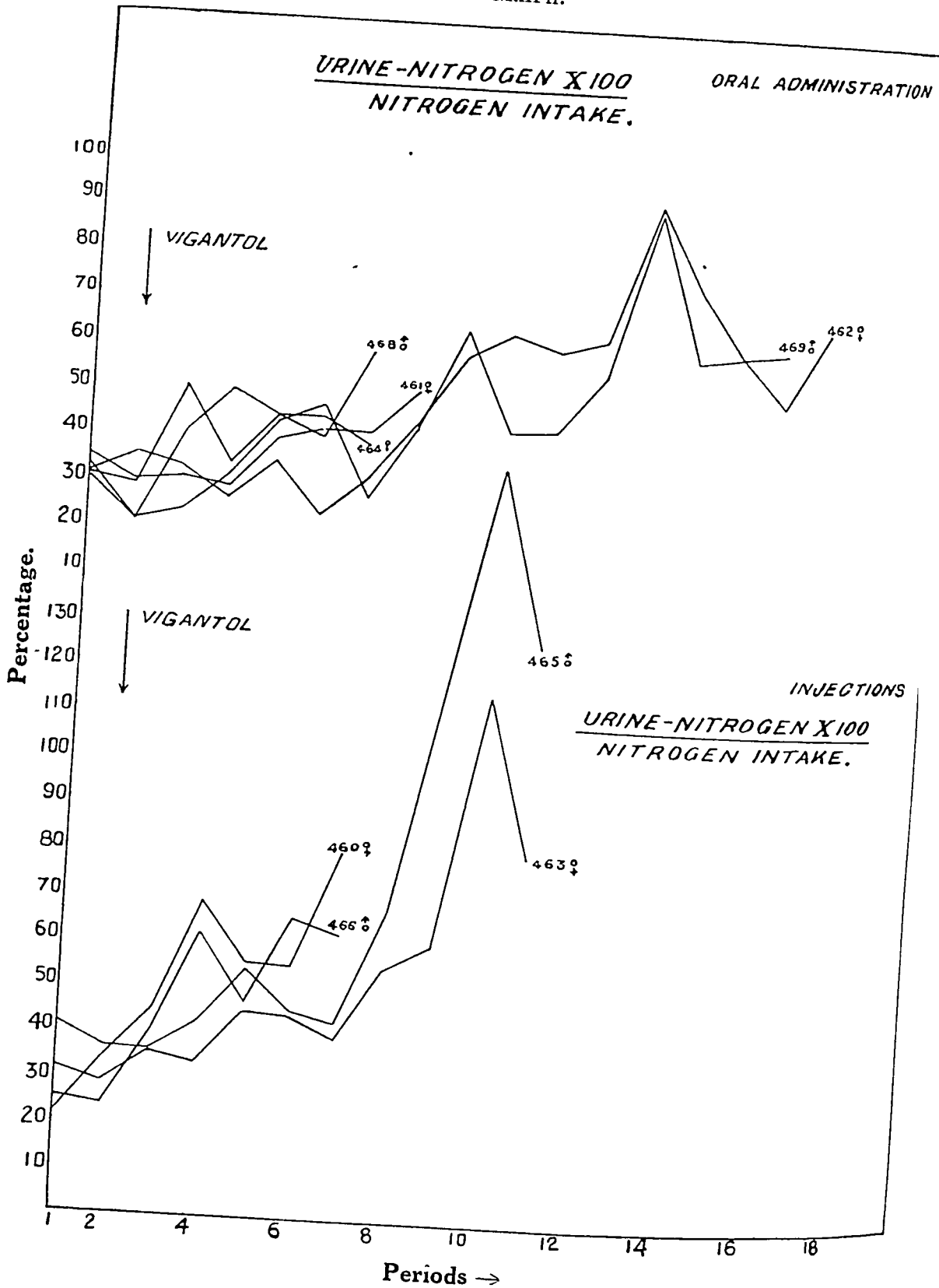
As is evident from the tables the effects of toxic doses of vitamin D have been brought out better in the injection group than in the oral administration group. It is possible that the orally administered vitamin D might not have been completely absorbed from the alimentary tract and the response to the toxic dosage came in evidence slower than in rats receiving Vigantol subcutaneously. Rat 462 showed no other symptoms of hypervitaminosis except that of a more gradual and less severe decrease in food intake than the rat 465; in the latter the fall in food intake once started went to the extreme limit. Similar results were obtained with other rats whose behaviour as regards the food intake followed either of these two depending upon the method of the administration of vitamin D. Kern, Montgomery and Still (*loc. cit.*) have stated that if the rats were given irradiated ergosterol separately, the adverse effect on food intake was little if any. The results presented in the tables obtained with as small a toxic dose as 4,500 I. U. per rat per day do not agree with the observations of the above-mentioned authors.

The effects on urinary nitrogen.—On administration of the toxic doses of vitamin D a rise in the urinary nitrogen was observed in rat 465 though no increase in the food intake had taken place. The high level of urinary nitrogen continued even after the food intake had decreased; it only came down after the latter had fallen to a very low level. This rat went into a negative nitrogen balance between the ninth and tenth periods. In rat 462 there was observed a fall in the urinary nitrogen after the commencement of excessive dosage with Vigantol. On comparing the urinary nitrogen with the nitrogen intake, however, the rise in the former became apparent. Rat 462 went into a negative nitrogen balance only temporarily, recovering later and remaining in a positive nitrogen balance till it was killed at the end of the experimental period.

So far as the increase in the urinary nitrogen is concerned it will be interesting to know whether it was due to an increased absorption from intestines or due to a simultaneous increase in the creatinine excretion. Experiments are in progress which are designed to show whether it is the endogenous or the exogenous protein metabolism that is accelerated by hypervitaminosis D. In the Graph are given the values for urinary nitrogen for nine rats expressed as the percentage of the nitrogen intake.

The effects on the Ca and P excretion.—In rat 465 the urinary calcium and phosphorus rose as a result of the subcutaneous injection of toxic doses of vitamin D. The high level of Ca excretion in the urine was maintained in spite of the diminished Ca intake. The urinary Ca and P fell sharply only when the food intake came down to 10 to 20 per cent of the original level; the fall in urinary P, however, set in earlier and was much sharper. In rats receiving Vigantol orally the urinary Ca and P also rose, in these cases, however, there was no fall towards the end of the experiment even though the food intake had fallen down to about 40 per cent of the original level. Thus, it would appear that in mild hypervitaminosis D, manifested by a partial loss of appetite as in rats receiving vitamin D orally, the urinary elimination of Ca and P was independent of the actual amount of Ca and P intake within certain limits.

GRAPH.



The oral administration of toxic doses of vitamin D caused a fall in faecal Ca and P. In rats of the injection group this fall was considerably delayed, though the other symptoms of toxicity had appeared already. The authors find themselves unable to explain this discrepancy at this stage. In later stages the proportion of ingested Ca and P recovered as faecal Ca and P rose in both the groups, showing possibly the combined effect of a decreased absorption and increased excretion into the intestines. This rise in the orally administered group was observed before the appearance of what Harris and Innes (*loc. cit.*) call the break-down of the gut function as a result of a severe hypervitaminosis D setting in. For in this group the severity of the other symptoms was not apparent. The food intake had fallen by approximately 50 per cent, while the rats had lost little weight. Hence it appears possible that the gut function is affected probably before the severity of hypervitaminosis sets in, a possibility which requires further testing. It might be noted that a certain irregularity does exist in day-to-day amounts of faecal Ca and P, hence it might be argued that since the dosage with vitamin D although toxic was not very excessive, its oral administration coupled with an indifferent absorption from the intestines contributed to the irregularity observed in the faecal calcium and phosphorus.

The retention of both calcium and phosphorus have been adversely affected, that of phosphorus more than that of the former (Table III). While in the control rats the calcium retention was about 70 per cent, in the hypervitaminotic rats it was between 26 and 41 per cent. Phosphorus retention was about 56 per cent in the four control rats; it had fallen down to 5 to 15 per cent in the hypervitaminotic animals. Light, Miller and Frey (*loc. cit.*) have reported a similar greater elimination of phosphorus than of calcium in hypervitaminosis D.

The effect on the demineralization of bone.—The rats receiving Vigantol were killed at the end of the experiment, their femora removed, carefully cleaned of the adhering tissue, and extracted with a mixture of ether and alcohol till free from fat. The bones were then dried and powdered. Weighed quantities of the bone powder were then ashed according to Stolte's method as described by van Slyke and Peters (1932), the ash dissolved in dilute hydrochloric acid and made to a known volume. Calcium and phosphorus were determined in aliquot portions by the methods described previously.

The femora of several normal animals were also analysed in a similar manner. The results of bone-ash analyses of the normal as well as the rats receiving toxic doses of vitamin D are given in Tables IV and V respectively.

The ash content of the bones of the normal rats is found to be higher than that of those receiving toxic doses of vitamin D. The demineralization of bone in hypervitaminosis D has been previously observed by Brown and Shohl (*loc. cit.*), and Harris and Innes (*loc. cit.*). Jones and Robson (*loc. cit.*), however, do not think that demineralization takes place under such conditions. They argue that the young growing rats, on which most of the experiments had been done by the earlier workers, suffer a loss of appetite on administration of toxic doses of vitamin D as a result of which Ca and P intake is reduced, which in turn affects the deposition of these elements in the bone. The control rats in the meantime, on account of the small doses of vitamin D, continue to eat well and grow normally

TABLE III.
Total intake and retention of Ca and P in controls and rats receiving irradiated ergosterol.

		* CONTROL RATS.				† RATS RECEIVING IRRADIATED ERGOS- TEROL.			
		350 ♀	355 ♂	359 ♂	361 ♂	462 ♀	469 ♂	463 ♀	465 ♂
Calcium	Intake, mg. ..	684.8	601.92	617.36	618.48	686.34	791.50	547.06	518.96
	Retention, mg. ..	528.32	433.92	462.00	451.04	226.50	209.08	215.72	208.58
	Retention, percentage ..	77.13	72.13	74.82	72.92	33.01	26.31	39.41	41.13
Phosphorus	Intake, mg. ..	547.52	495.52	477.44	487.76	554.46	639.48	441.70	418.74
	Retention, mg. ..	346.08	325.28	296.80	275.12	83.80	37.58	32.02	35.48
	Retention, percentage ..	63.22	65.51	62.18	56.40	15.11	5.88	7.25	8.47

* Figures for eight days.
† Figures for entire period of hypervitaminosis.

TABLE-IV.

Ca and P content of the ash of the femora of normal rats.

Rat number and sex.	Age in days.	Ash per cent on bone.	COMPOSITION OF ASH.		$\frac{\text{Ca}}{\text{P}}$
			Ca per cent.	P per cent.	
326 ♂ ..	200	58.88	36.39	16.29	2.23
328 ♂ ..	200	57.20	37.04	16.38	2.26
329 ♂ ..	200	60.40	37.20	16.44	2.26
332 ♀ ..	200	57.80	38.80	16.18	2.39
333 ♀ ..	200	59.10	37.70	16.60	2.27
336 ♀ ..	200	54.40	36.73	16.76	2.19
143 ♂ ..	300	62.09	38.28	17.02	2.24
142 ♂ ..	300	56.10	38.19	17.30	2.20
173 ♂ ..	300	59.70	36.95	16.28	2.27
338 ♂ ..	210	57.10	38.21	16.67	2.29
339 ♂ ..	210	60.20	38.37	18.20	2.10
341 ♀ ..	210	59.28	39.60	19.63	2.02
373 ♂ ..	180	57.10	39.26	18.24	2.15
374 ♂ ..	180	58.52	40.40	19.59	2.06
376 ♀ ..	180	58.30	38.50	16.62	2.31
350 ♀ ..	150	55.0	40.55	16.48	2.46
355 ♂ ..	150	55.66	39.17	16.79	2.33
359 ♂ ..	150	54.16	40.09	16.69	2.40
361 ♂ ..	150	58.90	38.73	17.14	2.26
3(B) ♀ ..	150	56.20	38.50	19.72	1.95
4(B) ♀ ..	150	54.80	39.20	18.10	2.16
3 ♀ ..	300	59.20	37.20	17.08	2.18
2 ♀ ..	300	58.10	38.10	17.15	2.22
Mean value	57.75	38.398	17.276	2.226
Standard deviation	2.202	1.178	1.098	0.115
Standard error	0.459	0.246	0.229	0.024

TABLE V.

Ca and P content of the ash of the femora of hypervitaminotic rats.

Administration of Vigantol.	Rat number and sex.	Age in days.	Ash per cent on bone.	COMPOSITION OF ASH.		Ca P
				Ca per cent.	P per cent.	
Orally for 13 days ..	461 ♀	193	61.60	37.76	13.74	2.75
„ 31 „ ..	462 ♀	203	56.36	38.53	14.71	2.61
„ 12 „ ..	464 ♀	222	51.36	38.20	14.07	2.71
„ 13 „ ..	467 ♂	193	57.15	37.90	15.52	2.44
„ 12 „ ..	468 ♂	222	47.94	37.81	14.91	2.53
„ 31 „ ..	469 ♂	203	50.75	38.08	15.38	2.47
Subcutaneously for 14 days	460 ♀	202	56.74	38.37	13.85	2.77
„ 28 „	463 ♀	237	59.16	38.33	13.58	2.82
„ 21 „	465 ♂	231	56.56	37.16	15.89	2.33
„ 14 „	466 ♂	202	57.15	38.11	15.70	2.42
Mean ..			55.477	38.025	14.735	2.585
Standard deviation ..			4.32	0.393	0.984	0.194
Standard error ..			1.367	0.124	0.311	0.0614

without an adverse effect on the mineralization of bone. At the time of the actual comparison the bones of hypervitaminotic rats naturally show smaller ash content than the bones of the rats receiving smaller doses of vitamin D. On these grounds Jones and Robson conclude that excess dosage with vitamin D has no specific effect on the demineralization of bone, but probably it interferes with the deposition of the bone salt itself. They further argue that provided their contention was correct there should be no reduction in the percentage of bone ash in adult rats receiving toxic doses of vitamin D. They in fact did not find a reduction in the ash content of the bones of adult rats under these conditions. The figures given in Tables IV and V were obtained by experiments on adult rats. These results indicate a demineralization of the bones in hypervitaminosis D.

A reference to the tables will show that whereas both the calcium and phosphorus content of the bone ash is reduced in hypervitaminosis D the extent of reduction is not the same with both of these elements. The observed difference between the mean values for Ca and P for the normal and hypervitaminotic rats is statistically significant. The effect of toxic doses of vitamin D seems to result

in a greater removal of phosphorus than of calcium, in consequence the Ca : P ratio changes from 2.226 ± 0.115 in the bone ash of the normal rats to 2.585 ± 0.194 in that of the hypervitaminotic rats. These results are not in agreement also with those of Kern, Montgomery and Still (*loc. cit.*) who reported no change in the Ca : P : CO₂ content of the bones of rats fed with toxic doses of irradiated ergosterol. The removal of extra phosphorus from the bones is in agreement with the observed excretion of phosphorus which is in excess of that of calcium.

These results might probably be said to have a bearing on the mineral composition of bone. That the latter is by no means constant for all ages even in the same species of animals has been shown by Kramer and Shear (1928). They showed that the value for $\frac{\text{carbonate Ca}}{\text{total Ca}}$ varied from 8 to 10 per cent in the normal young rats to about 15 per cent in the adult rats ; it was even greater in the rachitic rats. They indicated the possibility of the precipitation of a basic calcium salt in the freshly deposited bone salts. Robison (1936) has reviewed the evidence bearing on the mineral composition of bone from which it appears that various investigators are far from agreeing about the composition of the complex salt. Although it is generally agreed that the complex salt may be of the nature of the carbonato- or hydroxy-phosphate type, the possibility does exist of the presence of mixtures of calcium phosphate with other salts. Schmidt and Greenberg (1935) also visualize a similar possibility best expressed in their own words, '...it is clear that no single compound can account for the mineral matter in the bone. Even if in the main it is composed of a single complex salt it is probably admixed with other slightly soluble salts'.

These results can also be interpreted in a different manner. If the bone salts did exist as mixtures of calcium carbonate and calcium phosphate, both or either of these salts might be removed from the bone in the process of demineralization. In case only calcium phosphate was being removed the ratio $\frac{\text{total Ca}}{\text{P}}$ would increase. If such a supposition were correct the CaCO₃ : Ca₃(PO₄)₂ in the demineralized bone would also change. Further, there would remain no necessity to think in terms of a greater withdrawal of phosphorus than of calcium. Unfortunately the values of CO₂ content of the bone have not been determined in this series of experiments. Work is in progress dealing with these and other aspects of demineralization.

SUMMARY.

Vigantol was administered to adult albino rats orally and subcutaneously in doses of 4,500 I. U. per rat per day and the following observations made :—

1. The subcutaneous administration of Vigantol proved more toxic than the oral administration of an identical amount as judged by the loss of weight, loss of appetite, and the general condition of the rats.
2. The urinary nitrogen rose in spite of a stationary or diminished intake of food. It fell down sharply when the rats ceased to eat.
3. The urinary excretion of calcium and phosphorus increased and continued at a high level even during the period of the partial decrease in food intake.

4. Faecal calcium first fell and then rose in rats receiving Vigantol by mouth. The fall of faecal calcium was considerably delayed in rats to which Vigantol was administered subcutaneously.
5. Faecal phosphorus followed more or less faecal calcium, but was found to be considerably more irregular.
6. In rats receiving Vigantol more phosphorus was eliminated from the body than calcium. Both Ca and P retentions were reduced but that of P more so.
7. The ash content of the bones of rats receiving Vigantol orally as well as subcutaneously was less than that of the normal animals.
8. More phosphorus was withdrawn from the bones than calcium with the result that the Ca : P ratio was altered from 2.226 in the normal rats to 2.585 in the rats receiving Vigantol.

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URINARY COMPOSITION OF NORMAL BENGALEE SUBJECTS.

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UNDER normal conditions of diet and health, it is well known that an analysis of urinary constituents affords valuable information on the catabolic processes within the system and in fact on the whole problem of metabolism. It appears that no systematic work has yet been done on this subject in India. Observations on single specimens of urine, covering a period of only three to four hours, are quite common; but it will be understood that to correlate such results with total metabolism is open to serious criticism. The composition of urine varies from hour to hour during the day and only those results based on determinations covering a full period of twenty-four hours may be regarded as reasonably constant and to represent the true index of the metabolic activity of the individual.

The persons examined in this way all belong to the middle-class Hindu Bengalees. So far as could be ascertained, they had all been keeping in good health for the last two years. The age of the subjects ranges between 16 and 28 years. Members of both sexes are included so that these figures may be representative of the Bengalee middle class as a whole.

The specimens of urine were collected for 24 hours, a little toluene being used as a preservative. In order to prevent the loss of any ammonia, a little glacial acetic acid was added to the bottle before the collection of urine. This acid helped to keep the pH of the collected urine on the acid side. After 24 hours, the volume of the urine collected was measured and the estimations were made on an aliquot portion. The figures so obtained are given in the Table and represent grammes of each substance excreted during 24 hours. For comparison the corresponding figures for normal Americans and Europeans are placed side by side. The American figures are taken from Folin (1914), whereas the European figures are obtained from the book on 'Practical Physiological Chemistry' by Cole (1928).

TABLE.

Showing amount in grammes of each substance excreted during 24 hours.

	Bengalees*.		Americans.	Europeans.
Volume	1,700 c.c.	{ (Max. 2,100) (Min. 1,500)	1,500 c.c.	..
Urea	4.3	{ (Max. 6.58) (Min. 0.79)	31.55	30.0
Creatinine	1.38	{ (Max. 1.58) (Min. 1.09)	1.55	1.55
Uric acid	0.70	{ (Max. 0.90) (Min. 0.31)	0.64	0.70
Total nitrogen	4.83	{ (Max. 8.43) (Min. 2.89)	16.80	16.00
Inorganic sulphate (as SO ₃) ..	0.65	{ (Max. 1.80) (Min. 0.43)	3.27	2.92
Ethereal sulphate (as SO ₃) ..	0.10	{ (Max. 0.19) (Min. 0.00)	0.19	0.22
Neutral sulphate (as SO ₃) ..	0.18	{ (Max. 0.22) (Min. 0.12)	0.18	0.17
Chlorides (expressed as NaCl) ..	17.4	{ (Max. 21.3) (Min. 13.6)	10-15	..
Inorganic phosphorus ..	0.68	{ (Max. 1.11) (Min. 0.45)	0.85	0.90
Calcium	0.061	{ (Max. 0.067) (Min. 0.048)	0.17	0.15

* These figures are the averages based on the analysis of 24 hours' samples of urine of about fifty individuals.

Total nitrogen was calculated by the Kjeldahl's method, urea according to the urease method, creatinine according to Jaffe's method as modified by Folin (*loc. cit.*), uric acid by Benedict and Franke's (1922) method, sulphates by Rosenheim and Drummond's (1914) method, chlorides by Whitehorn's (1921) method, phosphorus by Fiske and Subbarow's (1925) method, and calcium by Shohl and Pedley's (1922) method.

DISCUSSION.

The most striking fact that is revealed in these analyses is the amazingly low values for total nitrogen, urea, and inorganic sulphates in the urine of normal Bengalees. Compared with these, the corresponding figures for average Europeans or Americans are five to seven times greater. These low values seem to prove that the average Bengalee diet is very poor in proteid food-matters. Direct measurements of the amounts of proteins ingested per day by middle-class Bengalees are now being investigated, but the results obtained so far fully corroborate the findings of the urine analysis. In this connection it may be of interest to compare the figures obtained by Folin in cases where the diet was known to be deficient in proteins. He found that such persons excreted on an average about 3.5 grammes of nitrogen, 4.72 grammes of urea, and 0.46 gramme of inorganic sulphate. It may be seen that these figures are remarkably similar to the figures obtained from Bengalee subjects.

It is well known that all metabolic nitrogen passes off in the urine, a negligible portion being lost in the fæces, perspiration, and epidermal substances such as hair and cuticle. It is customary to estimate the nitrogen of the fæces as one-tenth of the total nitrogen ingested. Calculated on this basis, i.e., by taking the total nitrogen in the urine to represent nine-tenths of the amount of nitrogen ingested with the diet, we find that an average Bengalee takes about 5.0 grammes of nitrogen per day; or, in other words, he is ingesting about 35 grammes of protein per day. This value confirms the previous findings of McCay (1908, 1911) and Campbell (1919) who gave the level of protein metabolism in the inhabitants of Bengal as between 30 and 40 grammes of protein per day. Now according to Chittenden, 'the physiological needs of the body are fully met by a metabolism of protein matter equal to an exchange of 0.10 g. to 0.12 g. of nitrogen per kg. of body-weight'. This would mean an allowance of 39 to 46 grammes of protein per day for a man of average weight (120 lb.). It must be noted that this figure of 39 g. to 46 g. of protein represents the minimum requirement and the amount which would ordinarily be allowed in planning a dietary is obviously much greater. But even disregarding this objection, it will be seen that the protein intake of an average Bengalee falls below the minimum. If the physiological protein minimum is taken to be 30 grammes, it appears that a middle-class Bengalee is existing on a diet which is supplying him with the barest minimum of proteid foods. Such a diet is absolutely detrimental to normal development and activities.

The complexity of these investigations is well illustrated in the following experiment. In one subject, the urinary nitrogen output was found to be 4.15 grammes per day. His diet when examined was found to contain 38.6 grammes of protein. The rice in the diet was changed to *atta*, thereby increasing the protein content by about 15 grammes. After the person had been on this diet for about a week, his urine was again examined and again found to contain 4.1 grammes of nitrogen, showing that the same amount of nitrogen as before was being metabolized. Unfortunately as we could not examine the fæces of the subject, we could not ascertain what amount of total protein was being absorbed. Wilson and Mookherjee (1935) noted that Bengalee subjects are unable to utilize the whole of the protein when put on a comparatively rich protein diet. This would mean that generation of low protein intake has so disorganized the alimentary

canal of the Bengalee subjects that it is not possible for them to break down and absorb more than a small quantity of protein at a time. The effect of habit on the absorption of food-materials has been little studied and it seems that more attention should be given to this point before any drastic change in the habitual dietary is advised.

The other figures, especially those relating to endogenous metabolism, i.e., the figures for creatinine and neutral sulphur excretions, are nearly the same for a Bengalee as well as for an American or European. The creatinine figures for the Bengalees are a little lower but that may be due to their smaller body-weights. This suggests that racial and climatic changes may not have a profound influence on endogenous metabolism.

The figures for uric acid are worthy of notice. It may be seen that the daily output of this acid by Bengalees or Europeans is nearly the same. This fact is rather curious because, the Bengalee diet being deficient in animal foods, the output of uric acid by Bengalees might have been expected to be much lower. Neither can this increase be due to mild hyperthyroidism, for Mukherjee and Gupta (1931) have shown that the basal metabolic rates for Bengalees are on an average 10 to 15 per cent lower than European figures. Our results tend to confirm those of Siven (1901) which showed that the greater part of the uric acid excreted is of endogenous origin. On the rice diet of Bengalees—a diet which tends to produce an alkaline urine—uric-acid production and elimination will be greatly stimulated. This stimulation in the endogenous formation of uric acid through the ingestion of a rice-rich diet coupled with the exogenous acid formed from the purines of fish, which forms a part of all Bengalee diet, may cause this apparently high concentration of uric acid in the urine of the Bengalees even though the diet is deficient in purine-rich materials such as liver, spleen, or thymus.

As regards the inorganic constituents of urine it will be seen that phosphorus figures for Bengalees, Americans, and Europeans are very nearly the same. As phosphorus in the urine is derived mainly from the casein in the diet, it appears that the amount of milk or milk products ingested by Bengalees or Europeans must be more or less equal.

Calcium output is definitely lower in the case of Bengalees. It is chiefly due to the deficient nature of their diet with respect to this element. Calcium in the Bengalee diet is supplied solely by milk, whereas the European or American diet is further enriched by the inclusion of cheese. As cheese is the richest source of this highly useful inorganic constituent, the daily intake of calcium by Europeans or Americans is much greater than in the case of Bengalees.

Chloride excretion is greater in the case of Bengalees. A comparison of the outputs of the normal Bengalees and the Americans shows that the former are excreting about 20 per cent more chlorides in the urine. These estimations were carried out during the months of April and June, when the weather was very hot and humid. In addition to the chlorides given out in the urine, the subjects must have lost a conceivable amount of chlorides in their sweat. Folin (*loc. cit.*) does not mention the weather conditions under which his experiments were conducted but from the date of publication of his paper, it appears that the experiments were conducted under winter and autumn conditions. It appears very probable,

therefore, that the average intake of salt by Bengalees must be about 50 to 100 per cent greater than that of Americans or Europeans. This point is of great interest and its significance is more fully discussed in another paper in which the metabolic activity of patients suffering from epidemic dropsy is studied.

SUMMARY.

The average composition of the urine of middle-class Bengalees in normal health is given. The averages are based on the analysis of a large number of normal urines collected over a period of twenty-four hours. It is found that, as compared with the corresponding figures for normal European or American subjects on ordinary mixed diets, total nitrogen, urea, inorganic sulphate, and calcium excretions are much lower, chloride excretion is definitely higher, whereas creatinine, uric acid, ethereal and neutral sulphate, and inorganic phosphate outputs are more or less the same. The significance of these findings is discussed.

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OBSERVATIONS ON THE METABOLIC ACTIVITY OF PATIENTS SUFFERING FROM EPIDEMIC DROPSY.

Part I.

URINARY COMPOSITION OF EPIDEMIC-DROPSY PATIENTS.

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In continuance of our work on the metabolic activity of the normal middle-class Bengalee (Ray and Ganguly, 1938), it was decided to extend our observations on urinary composition to pathological conditions. Epidemic dropsy formed the first of a series of observations, since it is well known that this disease is prevalent among the Bengalee community.

The methods of analysis and the collection of twenty-four-hour samples of urine were similar to those used previously. The Table gives the amount of the more common constituents present in the urine of epidemic-dropsy patients. For the sake of comparison, the corresponding figures for normal Bengalee subjects (Ray and Ganguly, *loc. cit.*) are placed side by side.

TABLE.

Showing amount in grammes of constituents present in the urine of epidemic-dropsy patients.

The results are all expressed in grammes per 24 hours.

				Epidemic dropsy.		Normal.
Urea	4.6	{ (Max. 6.33) (Min. 2.49)	4.3
Creatinine	1.22	{ (Max. 1.70) (Min. 0.85)	1.38

TABLE—concl'd.

			Epidemic dropsy.	Normal.
Uric acid			0.94 { (Max. 1.89) (Min. 0.49) }	0.70
Total nitrogen			4.56 { (Max. 7.33) (Min. 2.13) }	4.83
Inorganic sulphate			0.69 { (Max. 1.02) (Min. 0.07) }	0.65
Ethereal sulphate			0.10 { (Max. 0.29) (Min. 0.01) }	0.10
Neutral sulphate			0.17 { (Max. 0.25) (Min. 0.11) }	0.18
Chloride (expressed as NaCl) ..			11.4 { (Max. 16.8) (Min. 9.3) }	17.4
Inorganic phosphorus ..			0.11 { (Max. 0.22) (Min. 0.07) }	0.68

DISCUSSION.

It will be noticed that the figures for urea, total nitrogen, and inorganic sulphates are more or less the same as in normal subjects. Since most of the patients were more or less on the same diet which they were having before the onset of the disease, the similarity of the figures for the three excretas in normal and epidemic-dropsy patients would imply that protein metabolism on the whole is not much affected in epidemic dropsy. In more severe cases, where the diets have been restricted, the figures obtained were definitely different from those given in the Table; but the results of the urinary examinations of such severe cases have not been included.

The figures for creatinine and neutral sulphur are a little lower in the case of epidemic-dropsy patients. The difference, however, is not very significant and it may be partly due to the wasted condition of some of the patients.

Contrary to expectations, the values for ethereal sulphate was found to be the same in both normal and pathological subjects. In view of the prevalent idea that intestinal bacterial action may be one of the factors which cause epidemic dropsy, it might have been supposed that the amount of ethereal sulphates would have increased in epidemic dropsy, for it is well established that the organic sulphates owe their origin to the detoxication of cyclic-hydroxyl compounds arising from bacterial degradation of proteins and amino-acids in the intestine. It seems,

therefore, that, if epidemic dropsy is due to any bacterial toxin, it cannot be of phenolic nature. This fact is also supported by our observation that, except in one or two cases, indican could not be detected in the urine of epidemic-dropsy patients.

It will be noticed that the uric-acid excretion is definitely increased in epidemic-dropsy patients. This fact supports the previous findings of many observers that the uric-acid content of blood is also greatly increased in epidemic dropsy. The reason for this increased output of uric acid is at present obscure, but it probably points to the fact that the metabolism of the cell nuclei is greatly disturbed in epidemic dropsy. It may also be mentioned here that in three or four of the cases the thyroid gland was found to be definitely enlarged; but it is doubtful whether the increase in the uric-acid output is in all cases due to hyperthyroidism.

Another striking feature is the abnormally low excretion of inorganic phosphates in the urine of epidemic-dropsy patients. In fact, in our view, this finding may be utilized in the clinical examination of urine in suspected cases of epidemic dropsy. The average figure is five to six times lower than that of normal subjects and every case, which was definitely diagnosed as active epidemic dropsy, showed this lowered output. Even the maximum figure obtained was less than half the minimum figure obtained for middle-class Bengalees on an average mixed diet. In order to examine whether inorganic phosphates are accumulating in the blood, a few samples of blood of epidemic-dropsy patients were examined. The values were found to be only slightly higher than those for normal subjects. We had, however, the opportunity of examining two samples of pericardial fluids obtained from two subjects who had succumbed to this disease. The inorganic phosphate content found was 1.12 mg. and 0.87 mg. per 100 c.c., respectively. These concentrations are about four to five times higher than the phosphate content of lymph fluids. The fluids were, however, obtained from cadavers two days old, and unless cedematous fluids of living persons can be examined, we cannot positively assert that the lowered output of inorganic phosphates in the urine is due to the retention of such substances in the cedematous parts of the body.

A remark may here be made in connection with the excretion of chlorides. It has been pointed out in a previous paper (Ray and Ganguly, *loc. cit.*) that the average intake of salts by Bengalees is about 50 per cent higher than in the case of Europeans. It may be seen from the Table that the output of chlorides is definitely lower in the case of epidemic-dropsy patients. Obviously the patients are retaining a portion of the ingested salt, as in many of the cases examined the patients were taking the same diet as they had taken before they were attacked by this disease. The retention of chlorides is further proved by the fact that the concentration of chlorides in blood is increased during an attack of this disease. It is advisable, therefore, to decrease the amount of salt taken with the diet, in cases of epidemic dropsy. In this connection, it may also be noted that in the two samples of cedematous fluid examined, the concentration of chlorides was found to be similar to that of plasma.

In two of the cases, the calcium excretions were examined and were found to be 22.5 mg. and 17.2 mg. per 100 c.c. of the urine, respectively. These values are very small even when compared with the lowest values (61 mg. per 100 c.c.) recorded for normal Bengalee subjects. The observations were restricted to only

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two persons, because in all other cases the patients were taking extra calcium salts after the onset of the disease. In such cases, therefore, the calcium excretion does not indicate the true state of calcium metabolism within the body.

SUMMARY.

Examination of the composition of the urine of Bengalee patients suffering from epidemic dropsy has revealed the fact that the excretion of inorganic phosphates, chlorides, and calcium is very much lower than in normal Bengalee subjects. The figures for other urinary constituents are similar to those of normal subjects.

ACKNOWLEDGMENTS.

The authors wish to express their gratitude to Sir Nilratan Sircar for his kind interest in this research. Our thanks are due to Dr. C. C. Basu for his continual encouragement and advice and also for supplying us with the two samples of oedematous fluids. The expenses of this work were borne by a grant from the University of Calcutta.

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ADSORPTION OF HYDROGEN IONS BY SERUM GLOBULIN AND ITS ANTIBODY.

BY

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THE applicability of titration curves in studying the complex physico-chemical changes in the protein molecule due to immunization has been suggested from time to time. Thus, Marrack and Smith (1930) observed that the titration curve of diphtheria toxin-antitoxin floccule resembled that of normal serum pseudo-globulin. Biswas (1936) observed a differential adsorption of hydrogen ions from oxalic acid by the pseudo-globulins of normal and antidiphtheric serum. This may be accounted for easily by the assumption of certain changes, constitutional or otherwise, undergone by the globulin molecule during the process of immunization.

The object of the present paper is to study *in vitro* similar changes associated with the globulin molecule directly by selecting both as antigen and antibody a form of pure globulin. Accordingly, pure pseudo-globulin of normal horse serum and the antibody globulin prepared with the same as antigen by immunizing rabbits together with pure pseudo-globulin of normal rabbit's serum have been selected for the potentiometric titrations. Titration curves have been drawn with N/10 solutions of hydrochloric and oxalic acids, baryta, and caustic soda.

EXPERIMENTAL.

Normal horse serum was precipitated with half-saturation concentration of ammonium sulphate and the precipitate after centrifugalization dissolved in conductivity water, and dialysed under ether in a closed membrane till free from $(\text{SO}_4)''$ ions. The percentage of globulin in this solution was determined and adjusted to 0.5.

A 0.14 per cent solution of pure pseudo-globulin was also prepared from normal rabbit's serum by a similar process. Immunizing a rabbit with the former solution as antigen a serum was obtained with a precipitin titre of approximately 1 in

1,000. Pure globulin was prepared from this serum as before and a 0.44 per cent solution of pseudo-globulin was prepared.

Potentiometric titrations were carried out with hydroquinhydrone electrodes (Bjellmann and Lund, 1921) by using 10 c.c. of the globulin solution in a small titration vessel in an electrically controlled thermostat at $37.4^{\circ}\text{C.} \pm 0.1^{\circ}\text{C.}$ Titres corresponding to 1 decigram of globulin were calculated.

RESULTS.

TABLE I.

N/10 hydrochloric acid.

NORMAL SERUM PSEUDO-GLOBULIN.		ANTISERUM PSEUDO-GLOBULIN.	
c.c. of acid corresponding to 1 decigram of globulin.	pH.	c.c. of acid corresponding to 1 decigram of globulin.	pH.
0.0	5.20	0.00	5.39
0.4	4.25	0.45	4.55
0.8	3.41	0.90	3.55
1.4	2.66	1.59	2.71
2.0	2.33	2.27	2.23
2.8	2.02	3.17	1.92
3.6	1.86	4.08	1.78
4.4	1.76	4.99	1.65
5.2	1.68	5.90	1.57
6.2	1.55	7.03	1.50
7.2	1.52	8.17	1.42

TABLE II.
N/10 oxalic acid.

NORMAL SERUM PSEUDO-GLOBULIN.		ANTISERUM PSEUDO-GLOBULIN.	
c.c. of acid corresponding to 1 decigram of globulin.	pH.	c.c. of acid corresponding to 1 decigram of globulin.	pH.
0.00	5.20	0.00	5.39
0.40	4.28	0.45	3.96
0.80	3.55	0.90	3.32
1.40	3.05	1.59	2.87
2.00	2.62	2.27	2.57
2.80	2.30	3.17	2.31
3.60	2.10	4.08	2.14
4.40	1.99	4.99	2.00
5.20	1.92	5.90	1.94
6.20	1.86	7.03	1.87
7.20	1.76	8.17	1.81

TABLE III.

N/10 baryta.

NORMAL SERUM PSEUDO-GLOBULIN.		ANTISERUM PSEUDO-GLOBULIN.	
c.c. of alkali corresponding to 1 decigram of globulin.	pH.	c.c. of alkali corresponding to 1 decigram of globulin.	pH.
0.00	5.20	0.00	5.39
0.40	6.16	0.45	6.34
0.80	6.65	0.90	6.99
1.20	6.86	1.35	7.31
1.60	7.09	1.80	7.54
2.40	7.41	2.70	7.77
3.20	7.53	3.60	7.93
4.00	7.62	4.50	8.08
4.80	7.79	5.40	8.19
6.00	7.87	6.75	8.47
7.20	8.28	8.10	8.81

TABLE IV.

N/10 sodium hydroxide.

NORMAL SERUM PSEUDO-GLOBULIN.		ANTISERUM PSEUDO-GLOBULIN.	
c.c. of alkali corresponding to 1 decigram of globulin.	pH.	c.c. of alkali corresponding to 1 decigram of globulin.	pH.
0.00	5.20	0.00	5.39
0.44	6.86	0.49	6.32
0.90	7.00	0.98	7.41
1.34	7.22	1.47	7.67
1.80	7.57	1.96	7.79
2.70	7.79	2.94	8.00
3.60	7.88	3.92	8.18
4.50	8.06	4.90	8.34
5.40	8.26	5.88	8.42
6.30	8.34	6.86	8.65
7.20	8.41	7.84	8.81

TABLE V-a.

*Pseudo-globulin of normal rabbit's serum 0.14 per cent solution.**(a) N/10 hydrochloric and oxalic acids.*

N/10 HYDROCHLORIC ACID.		N/10 OXALIC ACID.	
c.c. of acid added per decigram of protein.	pH.	c.c. of acid added per decigram of protein.	pH.
0.00	5.03	0.00	5.03
1.42	3.11	2.84	4.72
2.84	2.67	4.97	4.14
4.97	2.25	7.10	3.62
7.10	2.05	9.94	3.26
9.94	1.91	12.78	2.93
12.78	1.78	15.62	2.62
15.62	1.70	18.46	2.40
18.46	1.61	22.01	2.18
22.01	1.55	25.56	2.07
25.56	1.50

TABLE V-6.

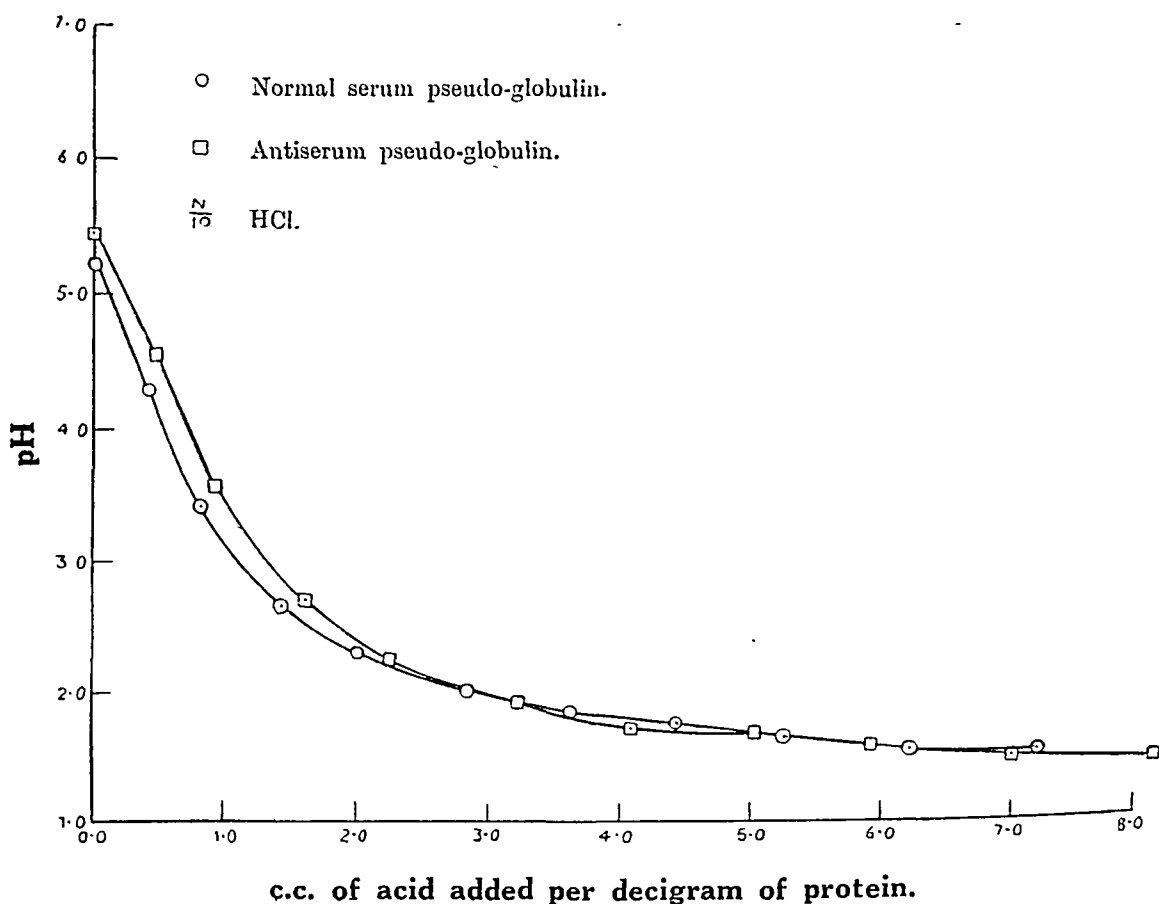
*Pseudo-globulin of normal rabbit's serum 0.14 per cent solution.**(b) N/10 baryta and caustic soda.*

N/10 BARYTA.		N/10 CAUSTIC SODA.	
c.c. of alkali added per decigram of protein.	pH.	c.c. of alkali added per decigram of protein.	pH.
0.00	5.03	0.00	5.03
1.42	5.75	1.42	6.94
2.84	7.10	2.84	7.43
4.26	7.41	4.26	7.66
5.68	7.61	6.39	7.82
8.52	7.82	9.23	8.10
11.36	7.98	12.78	8.28
14.20	8.26	15.62	8.39
17.04	8.47	19.17	8.65
21.30	8.83	22.01	8.86
25.56	9.14	25.56	9.04

DISCUSSION.

Graph 1 (*vide* Table I) represents the titration curves of normal serum (horse) pseudo-globulin and its antibody with N/10 hydrochloric acid. The results indicate only that the adsorption of H ions from a strong acid like HCl by both the globulins is practically identical below pH 5.0. Rigorously speaking,

GRAPH 1.

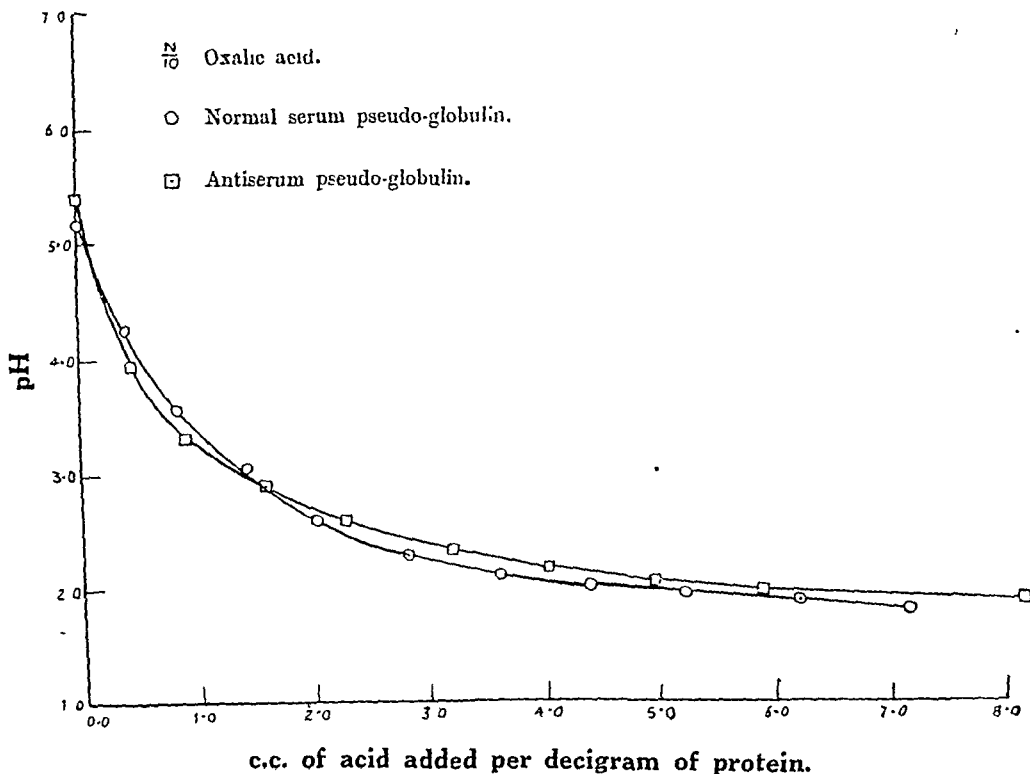


however, initial portions of the curves would suggest a superior capacity of adsorption of H ions by the antibody-globulin.

Graph 2 (*vide* Table II) describes the adsorption of H ions from oxalic acid. Here on the acid side of the iso-electric point of globulin at pH 5.3 (Michaelis and Davidsohn, 1912) no prominent difference of hydrogen-ion adsorption is observed

between the two globulins. Biswas (*loc. cit.*) observed with antidiphtheric serum globulin a characteristic difference of H-ion adsorption from oxalic acid as compared with that of normal globulin. Only near the iso-electric point (*vide* Graph 2) the curve of the antibody globulin is a bit steeper than that of the normal one.

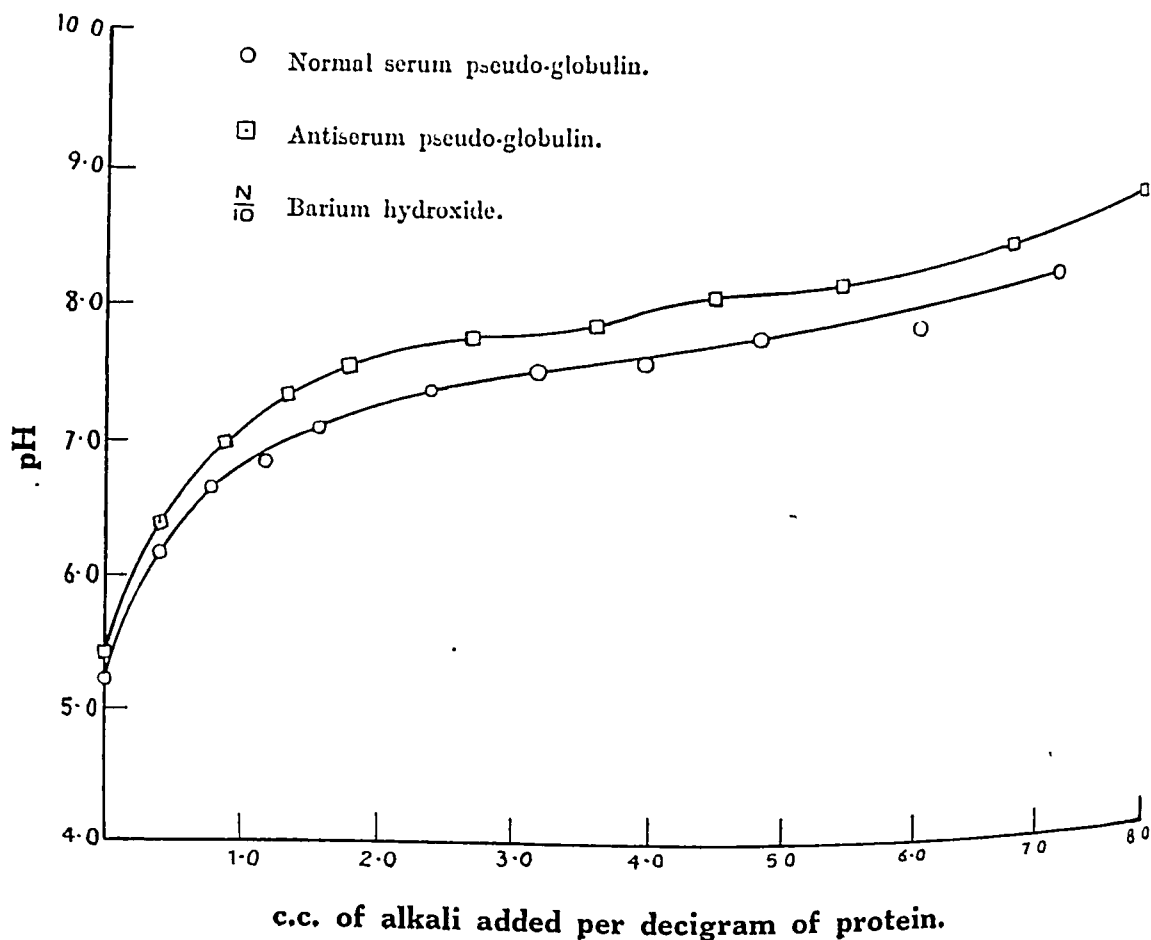
GRAPH 2.



Graph 3 (*vide* Table III) shows the behaviour of the globulins towards N/10 baryta. The adsorption curves run more or less parallel, indicating that titration

with a weak alkali is not influenced by the determinant groupings, if any, of the antibody globulin.

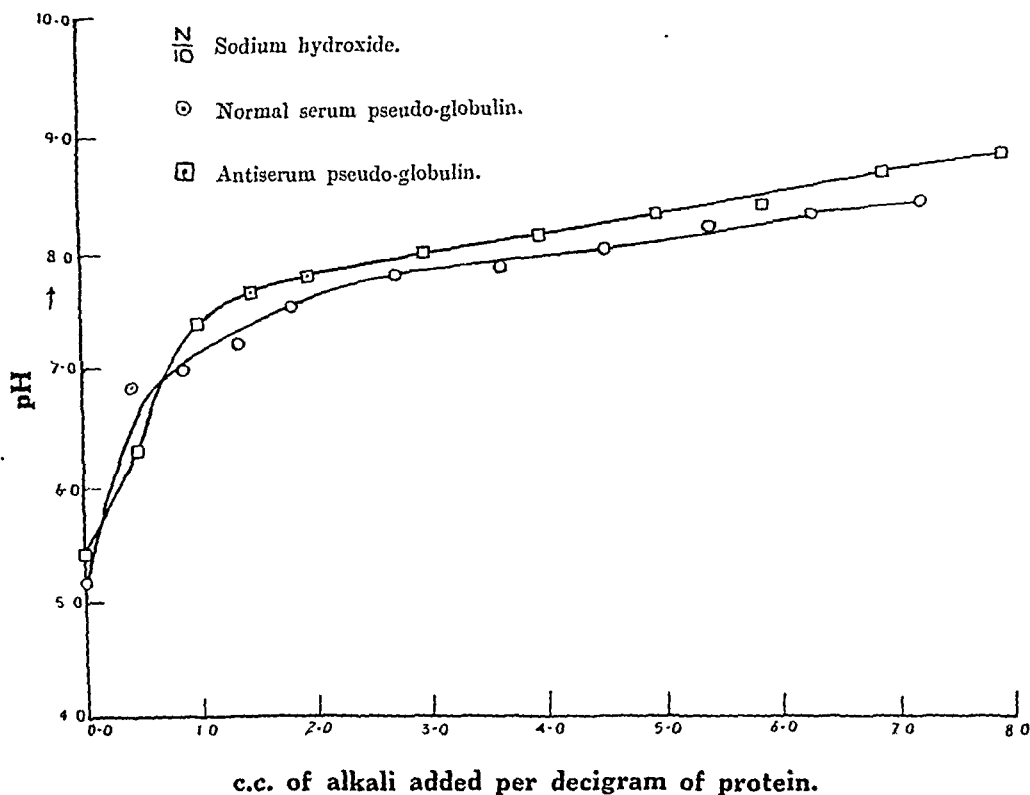
GRAPH 3.



Graph 4 (*vide* Table IV) represents the titration curves with N/10 NaOH. The curves intersect near about pH 7.0 and the steeper character of the normal globulin curve initially represents an inferior capacity of adsorption of $(OH)^-$ ions by the normal globulin which is equivalent to a superior affinity for H ions. This

is somewhat analogous to the observation by Biswas (*loc. cit.*) in the titration of antidiphtheric serum globulin at about pH 5.0.

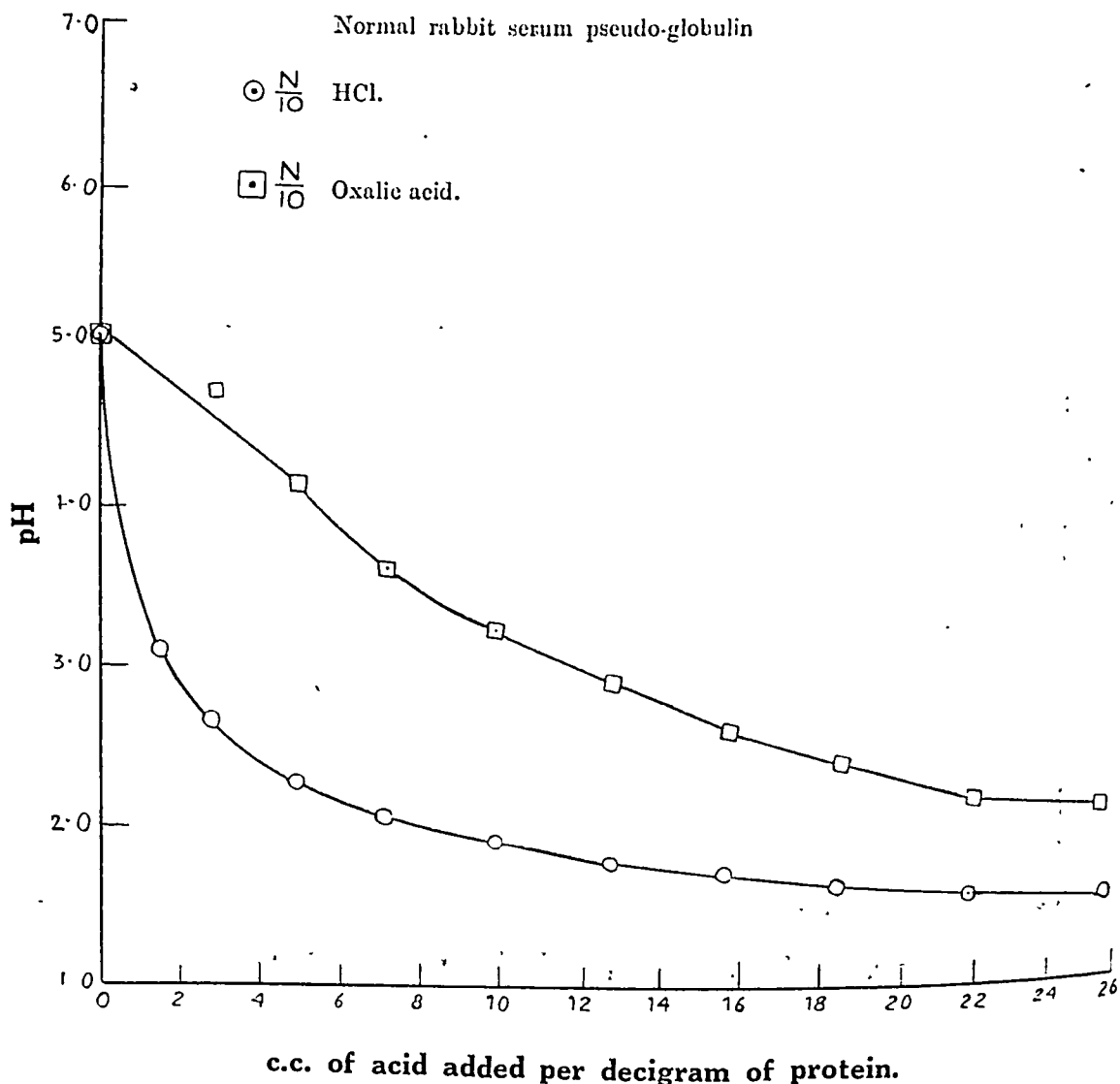
GRAPH 4.



Graphs 5-a and 5-b (*vide* Tables V-a and V-b) represent the titration curve of pseudo-globulin of normal rabbit's serum with decinormal solutions of hydrochloric and oxalic acids, baryta and caustic soda. These curves show parallelism with those of the pseudo-globulin of normal horse serum (*vide* Graphs 1, 2, 3, and 4). This would suggest that the results obtained with the normal globulin of horse serum

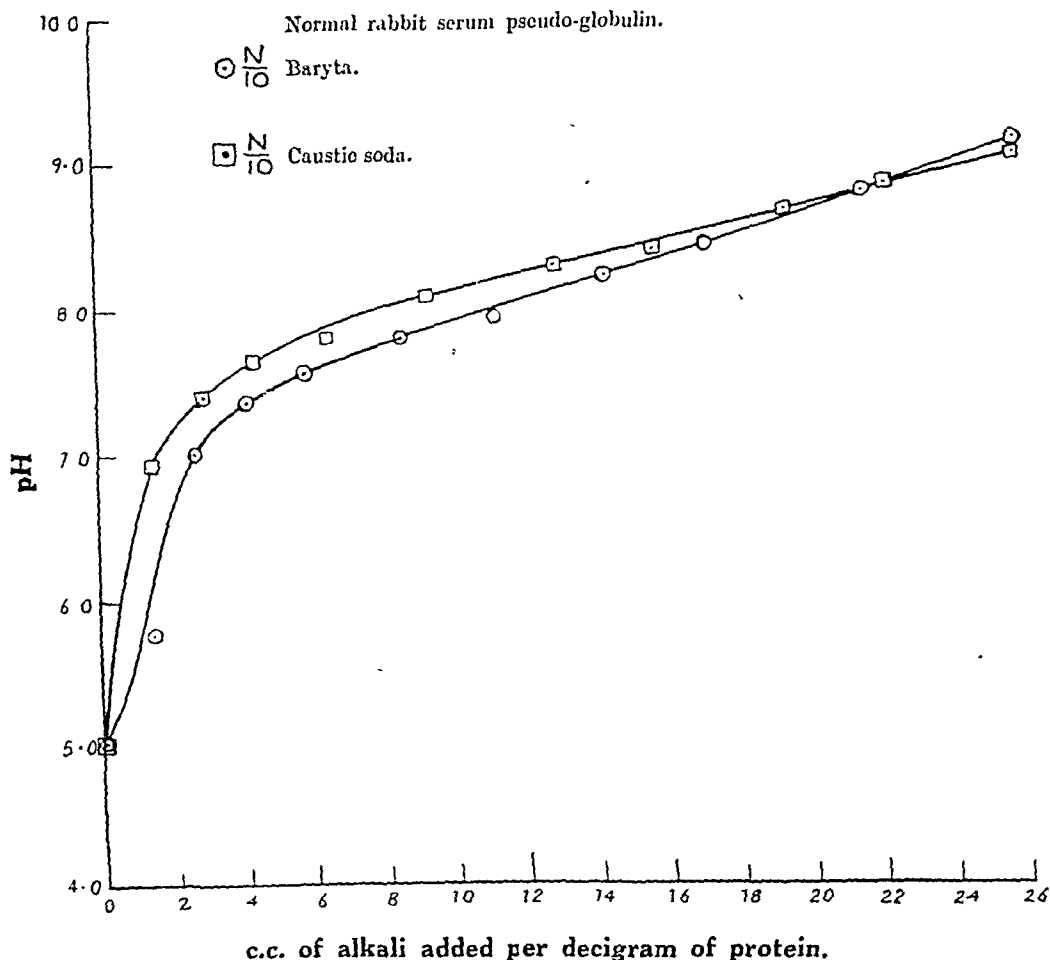
have also been corroborated in the case of normal rabbit's serum. Thus the observations with the immune globulin have been referred to both the normal globulins of horse serum, also used as the antigen, and of rabbit's serum.

GRAPH 5-a.



A general review of the titration curves would suggest that in the titration with a strong alkali like sodium hydroxide the characteristic groupings of the antibody globulin are probably brought into play at a higher level of pH than the iso-electric point.

GRAPH 5-b.



SUMMARY AND CONCLUSION.

1. Both the normal and the antibody globulins exhibit no marked difference in their capacities of adsorption of H ions from different acids, e.g., hydrochloric and oxalic.

2. Titration with a weak alkali, such as baryta, does not show any difference of H-ion adsorption between the two globulins.

3. Titration with a strong alkali, such as sodium hydroxide, suggests that normal globulin possesses a stronger affinity for H ions than the antibody globulin.

The study of titration curves thus helps considerably in making a qualitative survey of the physico-chemical changes undergone by the globulin molecule during immunization and it remains to introduce newer scientific methods in order to obtain quantitative results.

ACKNOWLEDGMENTS.

My best thanks are due to the Director of the Calcutta School of Tropical Medicine for granting me library facilities for my investigations, and to Mr. J. N. Lahiri, M.Sc., Manager, Bengal Chemical & Pharmaceutical Works, Ltd., Calcutta, for giving me active encouragement during the course of the experiments, but for which it would not have been possible for me to undertake this work.

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A PRELIMINARY NOTE ON THE CHEMISTRY AND PHARMACOLOGY OF THE LEAVES OF *SKIMMIA LAUREOLA*, HOOK, F.

BY

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Indigenous Drugs Inquiry, I. R. F. A.

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Skimmia laureola is an evergreen shrub of the Natural Order, *Rutaceæ*. It is known in Nepal as *Chumloni* and in the Punjab as *Ner*. It is distributed throughout the temperate Himalayas from Murree to Mishmi and Khasia mountains. It is a common shrub in the Doon Hills. The botanical description, which is given in detail in the 'Indian Medicinal Plants' (Kirtikar and Basu, 1918), is avoided here. The leaves are said to be burnt near smallpox patients for curative effects.

The species *Skimmia japonica*, Thunb., growing in Japan, has been examined in fair detail by different workers, such as Eijkman (1884), Schulze (1902), Honda (1904), and Asahina and Inubuse (1930). The leaves have been found to contain the alkaloid, *skimmianine* $C_{14}H_{13}O_4N$, *umbelliferon*, an essential oil containing a terpene, *skimmen* $C_{10}H_{16}$, and palmitic acid. The bark has been found to contain a non-toxic glucoside, *skimmin* $C_{15}H_{16}O_8$ and probably also *hesperidin*.

Some work has also been done with the leaves of the Indian species by such workers as Roure-Bertrand Fils (1920), Schimmel & Co. (1923), and Rajdhan (1930). These investigations have been confined to the essential oil, which has

been found to contain 13 per cent of terpenes (β -phellandrene, less of α -pinene), about 63 per cent of esters (mainly linalyl acetate), 18 per cent of alcohols (linalool, and probably some terpineol), azulene, an oil $C_{15}H_{26}O_2$, bergapten, and traces of an aldehyde and ketone.

There seems to be no published record of the other constituents of *Skimmia laureola* and the present investigation was undertaken to investigate these and to work out their medicinal value, if any. The dried leaves examined by us were kindly collected and identified by Mr. R. L. Badliwar from Gulmarg near Kashmir at about 8,000 to 8,500 feet above sea-level.

CHEMICAL EXAMINATION.

Preliminary test for alkaloids.—About 30 grammes of the powdered leaves were extracted with hot alcohol. The residue from the alcoholic extract was treated with dilute hydrochloric acid and the acid aqueous extract filtered. It was made alkaline and extracted with chloroform. The residue from chloroform was dissolved in dilute HCl and tested with alkaloidal reagents, such as Wagner's reagent, Meyer's reagent, Kraut's reagent, Sonnenschein's reagent, picric acid, and phosphotungstic acid. The tests were all strongly positive and showed the presence of fair amounts of alkaloids.

Assay for total alkaloids.—Twenty-five grammes of the powdered leaves were shaken with 200 c.c. of Prollius mixture in a stoppered cylinder for some time. One hundred c.c. of the extract were drawn off and evaporated. The residue was extracted with 1 per cent HCl and filtered. The filtrate was made alkaline with dilute ammonia and extracted repeatedly with chloroform. The amount of alkaloid obtained corresponded to 0.5 per cent of the dried leaves.

Preliminary systematic examination.—Fifty grammes of the powdered leaves were extracted in a glass Soxhlet successively with petroleum ether (B. P. 40°C. to 60°C.), ether, chloroform, and absolute alcohol. The solvents were removed in each case and the residues weighed and tested qualitatively for the nature of the constituents. Petroleum ether extracted 5.7 per cent; the residue was a green sticky mass and consisted mainly of chlorophyll, some essential oil, fatty oil, and waxy matter. Ether extracted 2.66 per cent, the residue consisted of traces of essential and fatty oil. Chloroform extracted 1.2 per cent; the residue was a brownish mass which gave feeble reaction for alkaloids. Finally, absolute alcohol extracted 17.4 per cent; the residue was a brownish viscous mass. Besides alkaloids, it gave reactions for sugars, organic acids, and traces of glucosides.

with small quantities of cold absolute alcohol when an oily portion was removed leaving the alkaloid in a crystalline condition. The residue was re-crystallized from hot absolute alcohol when it was obtained as yellow, rhombic, octahedral crystals melting at 175°C. to 176°C. The yield of the pure alkaloid amounted to 2.5 grammes.

The alkaloid was insoluble in petroleum ether, sparingly soluble in ether and cold absolute alcohol, soluble in hot alcohol, and readily so in chloroform. It dissolved in dilute mineral acids only when they were present in large excess. In HCl solution, it gave voluminous precipitates with alkaloidal reagents. The platinic chloride crystallized in rhombic plates and decomposed at about 185°C. Crystals of the pure base when treated with concentrated HNO_3 gave a yellow solution which changed to orange red. With concentrated H_2SO_4 it formed a brownish yellow solution which on the addition of KClO_3 became reddish brown.

From all the physical and chemical properties studied there could be no doubt that the alkaloid isolated from *Skimmia laureola* and purified as above was identical with *skimmianine* isolated before from *Skimmia japonica*.

Attempts to isolate a glucoside.—Eijkman (*loc. cit.*) isolated a glucoside from *Skimmia japonica* which he designated as *skimmin* $\text{C}_{15}\text{H}_{16}\text{O}_8$, and which is stated to resemble *æsculin* and *scopolin* very closely in its properties. Some of the methods for the isolation of glucosides were tried but none could be isolated from this species. Owing to the sudden departure of one of the workers (R. G. C.) further chemical work on the drug had to be postponed.

Pharmacological action of skimmianine.—Honda (*loc. cit.*) first tested the action of *skimmianine* and found that its injection into the femoral lymphatics of *Rana esculenta* or *Rana temporaria* affects the appearance of the muscles at the place of application, and renders them stiff and brittle. The neighbouring muscles are also affected by larger doses. Voluntary motion becomes sluggish, the breathing becomes shallow, and the pupils get contracted. Reflex sensibility appeared as a rule to increase only in the case of *Rana esculenta*. The absolute strength and the work performed by the muscles were apparently diminished. The alkaloid has probably a direct action on the muscles of the heart, decreasing the amplitude of systolic contraction and causing disturbances of the diastole. The pulse is similarly affected even when atropine has been previously administered. Intravenous injection in rabbits causes general symptoms of poisoning. With moderate doses, muscular spasms are usually noticed. There is an initial fall of blood-pressure but the ultimate effect is a rise probably due to peripheral vasoconstriction. *Skimmianine* has no effect on the secretion of urine.

Honda stated that the alkaloid could only be dissolved by dilute mineral acids when present in excess and he has probably carried out his experiments with the acid solution. We experienced the same difficulty in trying the pharmacological action. It was soluble in dilute HCl when the acid was present in some excess. The reaction was near about pH of 1.3 and in trying to neutralize the solution further, the alkaloid was precipitated. The pharmacological action of this solution was tried on rabbits, cats, and frogs. The action of the acid, however, was so predominant that it masked the action of the alkaloid to a great extent. Other salts of the alkaloid were tried but none of them yielded any more soluble

neutral solution. With the present state of our knowledge, it is not, therefore, possible to ascribe any specific pharmacological action to this alkaloid.

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SOME INORGANIC PREPARATIONS OF THE INDIAN INDIGENOUS MEDICINE.

Part VI.

SAMUDRA PHENA.

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Indigenous Drugs Inquiry, I. R. F. A.

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THE name *Samudra phena* is derived from the words *Samudra* (sea) and *Phena* (foam), as it is generally believed to be the dried foam of sea water. In reality, it is the calcareous shell of a sea fish, probably of *Sepia officinalis*. The shell is oblong or elliptical and is very hard and brittle. The outer surface is smooth and composed of thin flat pieces about $\frac{1}{2}$ mm. in thickness arranged one above the other in a heap of thin layers, each layer being separated from the other by longitudinal ridges. It can be easily scratched and pulverized. The inner surface is hard, porous, and easily friable.

Samudra phena is used in Ayurvedic medicine as well as a very common household remedy. In earache and oedema around the external auditory meatus a paste made with *Samudra phena* and the juice of *Datura fatuosa* is said to be highly beneficial. A powder made from *Samudra phena* is also dusted into the ear to relieve earache and otorrhoea. A medicated oil is prepared by boiling its fine scrapings in sesame oil which is used in earache. In skin diseases, it is applied locally with

lime-juice and with rose-water, it is applied to the body in prickly heat. According to a well-known Ayurvedic physician of Calcutta who had kindly supplied us with a genuine specimen of the drug for our analysis, it is a rich and cheap source of organic calcium and is used both externally and internally. In Ayurvedic practice, its internal use is not commonly recommended, but he has found it to be very effective internally and he considers it to be better than calcium lactate. The dose is from 5 to 15 grains.

The sample sent for analysis was a white lump, elliptical in shape. It was finely powdered and intimately mixed before making the qualitative and quantitative analyses. The result of the quantitative analysis is as follows:—

	Per cent.
Lime, CaO	49.725
Silica, SiO_2	0.580
Iron, Fe_2O_3	0.324
Alumina, Al_2O_3	0.102
Phosphoric acid, P_2O_5	0.048
Carbon dioxide, CO_2	38.560
NaCl	1.670
Potash, K_2O	Trace
Magnesia	Trace
Sulphates	Trace
Moisture	3.925
Organic matter	5.066
TOTAL	100.000

The amount of nitrogen in the organic matter amounted to 0.364 per cent of the total. Of the total calcium present, 49.076 per cent is combined with carbon dioxide as calcium carbonate and the balance 0.649 probably as organic calcium.

PHARMACOLOGY, THERAPEUTICS, ETC.

Being an insoluble inorganic preparation, the pharmacological action could not be studied in the usual manner. From the nature of the constituents, it is expected to be of some use clinically, both externally as an astringent and internally where calcium is indicated. An impure and uncertain product like this does not, however, offer any special advantage over several pure salts of calcium now being used in medicine.

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STUDIES ON THE PROTEASE OF COBRA VENOM.

BY

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THE application of cobra venom in therapeutics is of recent origin. Calmette *et al.* (1933) and Tagueet and Rouseau (1933) have found that cobra venom injected into spontaneous or grafted tumours of mice had a marked curative effect. Analogous results were obtained from intra-mural injection of cobra venom in certain malignant tumours. Cobra venom is a complex substance but the enzymes responsible for its effects on the tumour cells are, in the order of their importance, (1) phosphodiastase, (2) proteolysin, (3) hæmolysin, and (4) possibly hæmorrhagin. The venom introduced into the tumour forms with the lecithin of the cells a lysocytin which has a pronounced cytolytic effect. In addition, the action of the proteolytic enzyme always present in the tumour cells is augmented by the proteolytic enzyme of the cobra venom.

Chopra, Mukherjee and Chowhan (1937) have reported that both viscosity and surface tension of the blood suffer changes on injection of cobra venom, indicating a probable rise in protein concentration in the plasma. This rise in protein concentration is temporary and, in about 24 hours' time, the blood appears to assume normal values. The authors have not given an explanation how the viscosity and the surface tension are restored to their normal range. It is probable that the proteolytic enzyme of the venom comes into play and digests the extra proteins. If this were so, the protein concentration may even be expected to decrease in the course of a few days, as the enzymic digestion of the blood proteins proceeds. The rôle of the proteolytic enzyme in the venom, therefore, appears to be of great significance.

In the literature concerning the nature and action of the protease in cobra venom, there appears to be no record of a systematic study of factors affecting the earliest stage of protein break-down by this enzymic complex. Ganguly (1936) has confined himself to the detection of enzymes present in cobra venom without making any attempt to study the nature of the same. Ghosh *et al.* (1936a and b) in their studies on the enzymes of the venom have obtained some evidence regarding the nature of the proteolytic enzyme in the venom. They have recorded that the optimum activity of the proteolytic enzyme lies between pH 7.6 and pH 8.2 depending on the nature of the substrate used and that the enzyme resembles trypsin. The methods employed by these authors involve estimation of the COOH groups set free in the process of hydrolysis, and the experimental data obtained by them, while throwing considerable light on the nature of the enzymes, require, in our opinion, to be supplemented, to establish the true nature of the enzyme. Systematic examination of the kinetic behaviour of the enzyme action on a suitable substrate should lead to a better understanding of the enzyme and to a correct estimate of the true enzymic activity.

All the known proteases can be characterized by their pH optima and activation behaviour. The optimum pH for pepsin is near 2.0, for cathepsin 4.0, for pancreatic protease 9.0, and for papain and yeast proteinase about 5.0. Marked activation by HCN is the rule with most proteases of the cathepsin type from animal tissues or of the papain type from vegetable tissues. A careful study of these factors will throw light on the nature of the proteinase present in the venom. The present paper embodies the results of such a study.

EXPERIMENTAL.

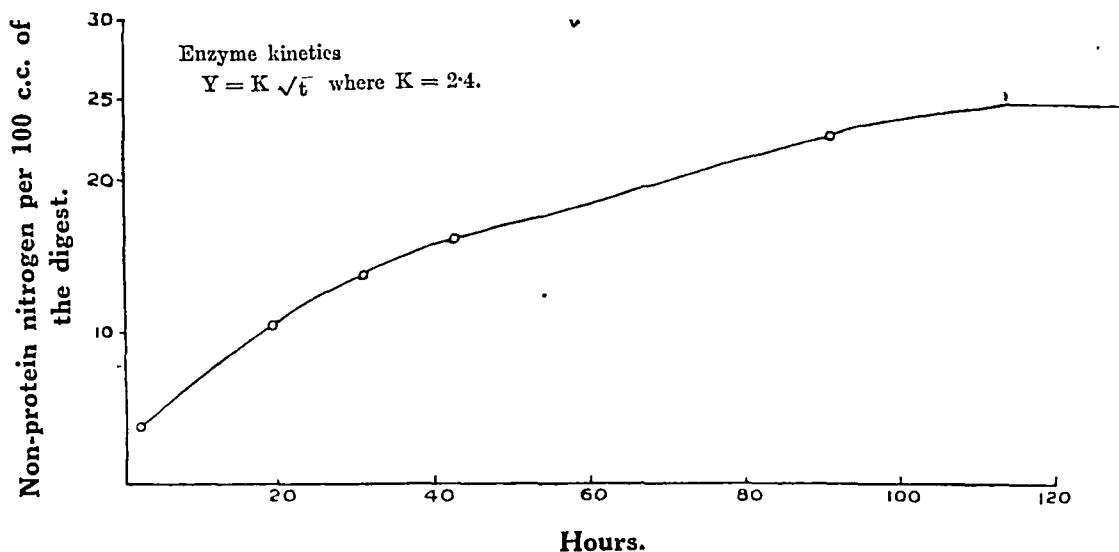
The lowest concentration of cobra venom with measurable enzyme activity was found to be 0.05 per cent (1 in 2,000). This concentration was chosen for the study of the kinetics with a view to approach as far as possible therapeutic doses.

Estimation of proteolysis.—The digestion of the protein was followed by the estimation of non-protein nitrogen soluble in trichloro-acetic acid after incubation of the enzyme solution with casein in buffered solution. For this estimation, the method of incineration followed by distillation of the ammonia into standard acid and back titration with CO₂ free N/500 NaOH, was employed. This procedure permitted estimation of the non-protein nitrogen to the nearest mg. per 100 c.c. of the digest. Two c.c. of the digest were precipitated with 5 c.c. of 10 per cent trichloro-acetic acid. The precipitated protein was filtered off and 5 c.c. of the filtrate taken for non-protein nitrogen estimation. A control experiment with heat-inactivated enzyme solution, and casein in similar concentration, was also carried out simultaneously. A method of this type offers advantages chiefly in respect of the applicability to the earliest stages of the protein digestion, over Willstätter's method involving the estimation of free amino group.

Enzyme kinetics.—Five c.c. of a 0.2 per cent solution of cobra venom were added to 50 c.c. of a 1 per cent solution of the sodium caseinate in buffer of pH 8.6, and incubated at laboratory temperature. The non-protein nitrogen of this solution was estimated at the start and at intervals till it reached a constant value.

The digest was saturated with chloroform to prevent bacterial contamination. The results of the experiment are shown in Graph 1. It will be seen that the amount of protein digested could be represented by the formula $Y = K\sqrt{t}$, where 'Y' is the increase in non-protein nitrogen, and 't' is the duration of the digestion. Within the limits of experimental error, the observed points are in agreement with the curve represented by the equation $Y = K\sqrt{t}$, where K is given the value 2.4. The final value of non-protein nitrogen represents only 40 per cent hydrolysis. The enzyme in such low concentrations appears to undergo inactivation.

GRAPH 1.



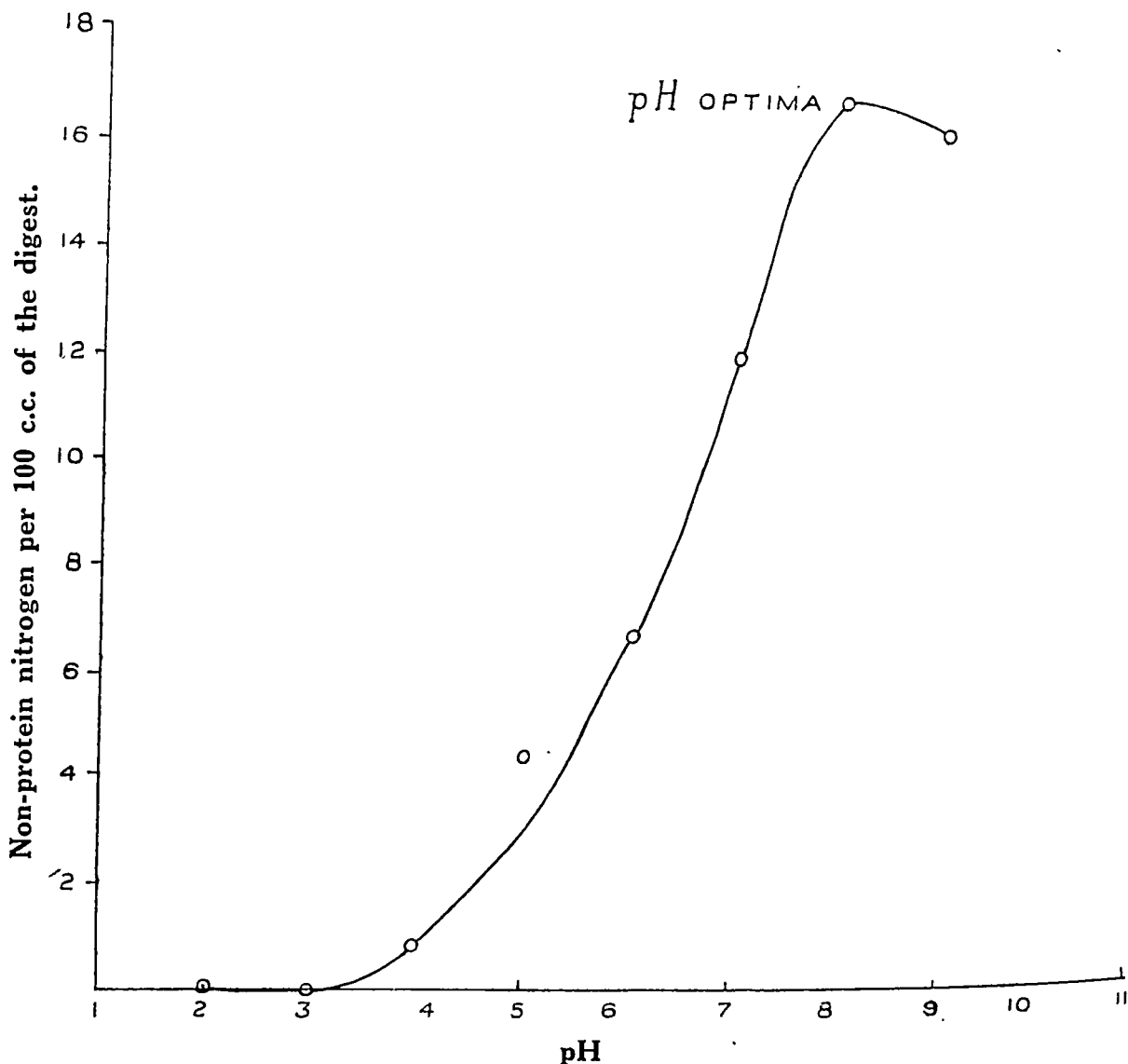
DETERMINATION OF THE OPTIMUM pH FOR PROTEOLYSIS.

A series of buffer solutions at pH 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0 were prepared using the respective salts required for each range. The buffered substrates for these experiments were prepared as follows:—

Five c.c. of a 2 per cent casein solution were transferred to a graduated cylinder and the pH adjusted to the required value with decinormal sulphuric acid or decinormal sodium hydroxide. To this solution 2 c.c. of the buffer of the same pH were added and the volume made up to 9 c.c. This solution was transferred to a test-tube to which 1 c.c. of the freshly prepared enzyme solution was added, thus adjusting the concentration of the substrate to one per cent. Initial samples were taken for estimation of non-protein nitrogen and the digest then incubated for 24 hours at the laboratory temperature. At the end of this period 2 c.c. from each digestion tube were taken and precipitated by the addition of 5 c.c. of trichloroacetic acid and the non-protein nitrogen in the filtrate estimated in the manner

described above. The results are represented in Graph 2. The optimum pH is thus found to be in the neighbourhood of 8.0.

GRAPH 2.



The behaviour of the enzyme towards small concentrations of HCN was next investigated. To 5 c.c. of 2 per cent casein solution 2 c.c. of 0.1M solution of HCN and 1 c.c. of enzyme solution were added and the volume made up to 10 c.c. The concentration of the HCN in the digest was 0.02M. The digest was incubated for 24 hours at the laboratory temperature, at the end of which period the released

non-protein nitrogen was estimated. The control experiments recorded in the following tables were also carried out simultaneously:—

Experiments.	Increase in non-protein nitrogen (mg. per 100 c.c. of the digest).		
(1) Untreated	11.5
(2) Treated with HCN	8.5
(3) Heat-inactivated control	0.4
Inactivation by HCN	26 per cent

A similar experiment to the above using 0.1M solution of KCN was carried out. The final concentration of KCN in the digest was 0.02M. The quantities of substrate and the enzyme were exactly similar to those in the previous experiments.

(1) Untreated	11.2
(2) Treated with KCN	10.1
(3) Heat-inactivated control	0.5
Inactivation by KCN	10 per cent

DISCUSSION.

The demonstration of the optimum pH at 8.0 for hydrolysis of casein by the venom and its behaviour towards KCN and HCN show that the protease in cobra venom belongs to the group of tryptases, and that papainases and pepsinases are absent. The amount of the casein digested is proportional to the square root of the duration of digestion in accordance with the relationship inunciated by Arrhenius with regard to the tryptic digestion of gelatin and casein.

Studies in the enzyme kinetics show that the concentration of the proteolytic enzyme in the venom is small and during the digestion, the trypsin inhibitor which appears to be present in the venom checks the further hydrolysis of the substrate. The presence of the zinc salts reported in the literature, and further confirmed by us with the help of spot tests, might be associated with the trypsin inhibitor. This interesting finding of the presence of a trypsin inhibitor is significant in view of the reports of its efficacy in the treatment of cancer. The low concentration of the venom combined with the presence of the agent to inhibit the digestion after it attains a certain stage, might be the factors responsible for the selective digestion of the tumour cells without affecting the original tissue. The studies of the other factors responsible for the effects reported in literature are in progress.

SUMMARY.

1. The rate of proteolysis of one per cent casein solution by cobra venom in the concentration of 0.05 per cent has been studied by estimation of non-protein nitrogen released at definite intervals. The amount of casein digested is proportional to the square root of the duration of digestion.

2. In lower concentrations, the enzyme is gradually inactivated during digestion due possibly to the presence of a trypsin inhibitor.

3. The optimum pH for the digestion of casein is found to be approximately 8.0.

4. The protease is inactivated by HCN and KCN.

5. The proteolytic enzyme of cobra venom appears to belong to the group of tryptases. The significance of the presence of the protease in the venom and its therapeutic applications are discussed.

ACKNOWLEDGMENT.

The authors wish to express their grateful thanks to Brevet-Colonel R. N. Chopra, C.I.E., K.H.P., I.M.S., for his suggestion of the problem, for his constant advice during the course of the investigation, and for his kind permission to publish this paper.

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CULTIVATION OF THE RABIES VIRUS ON THE CHORIO-ALLANTOIC MEMBRANE OF THE DEVELOPING EGG.

BY

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AND

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ATTEMPTS have been made to cultivate the rabies virus on the chorio-allantoic membrane of the developing egg, but the results have been contradictory. Waldhecker (1935) reports that the rabies virus cannot be successfully cultivated on the chorio-allantoic membrane. Schultz and Williams (1937) mention that the attempts to propagate the virus on the chorio-allantoic membrane yielded entirely negative results. On the other hand, McKendrick (1937, 1938), in a review of the recent articles on rabies, mentions that Peragallo has been successful in cultivating the virus.

The present-day antirabic vaccine is a crude suspension of a 5 per cent fixed virus brain tissue in half per cent carbol-saline. If attempts at the cultivation of the rabies virus on the chorio-allantoic membrane prove successful and if the virus so cultivated retains its immunizing properties, it would be possible to prepare a vaccine free from the brain tissue, which is regarded by some as the ætiological factor in the production of ascending paralysis after antirabic treatment. Further, the cost of production would be cheaper, and perhaps it would be possible to prepare a more concentrated vaccine.

A series of experiments were, therefore, undertaken to verify if the virus could be successfully cultivated.

EXPERIMENTAL PROCEDURE.

Virus.—Inoculated animals showing signs of rabies were killed and their brain removed aseptically. The brain of each animal was weighed and ground up with a measured quantity of Tyrode solution. This suspension was centrifugalized and the supernatant pipetted out, tested for sterility and used for inoculation into

eggs. Varying concentrations, 5 to 20 per cent. of both fixed virus and street virus were used.

Eggs 12 to 14 days old were candled to determine the viability of the embryo and used in the experiment. With the aid of a strong source of light for transillumination, the position of the air sac was marked out. To ensure sterility of operation, the inoculation of eggs was carried out inside a glass chamber, which was cleaned daily with an antiseptic before operation.

A square portion of the shell, about 1 cm. wide, over the air sac lying just above its lower limit was cut out by using a sharp file. The egg was kept in a holder inside the glass chamber and the area swabbed with 5 per cent carbolic acid and then with absolute alcohol. The square piece of shell was removed, and the outer shell membrane was cut out with a sterile cataract knife avoiding trauma to the inner shell membrane. The chorio-allantoic membrane was next inoculated with the virus according to the technique described by Brandly (1935). To facilitate the introduction of the inoculum immediately beneath the inner shell membrane and above the chorio-allantoic membrane, the bevel of the needle was turned down and kept almost parallel to the inner shell membrane. A slight elevation and wetting of the inner shell membrane showed that the inoculation had been satisfactory. 0.1 c.c. of the inoculum was used throughout. To facilitate observation the shell opening was closed after inoculation by means of a disc of colourless cellophane paper swabbed with alcohol and applied with sterile library paste. The eggs were then incubated for three days at 39°C.

The inoculated eggs were candled twice daily. Darkening and immobility, paleness or disappearance of the blood vessels, and the development of a grey colour of the inner shell membrane, which is visible through the cellophane window, indicate that the embryo is dead.

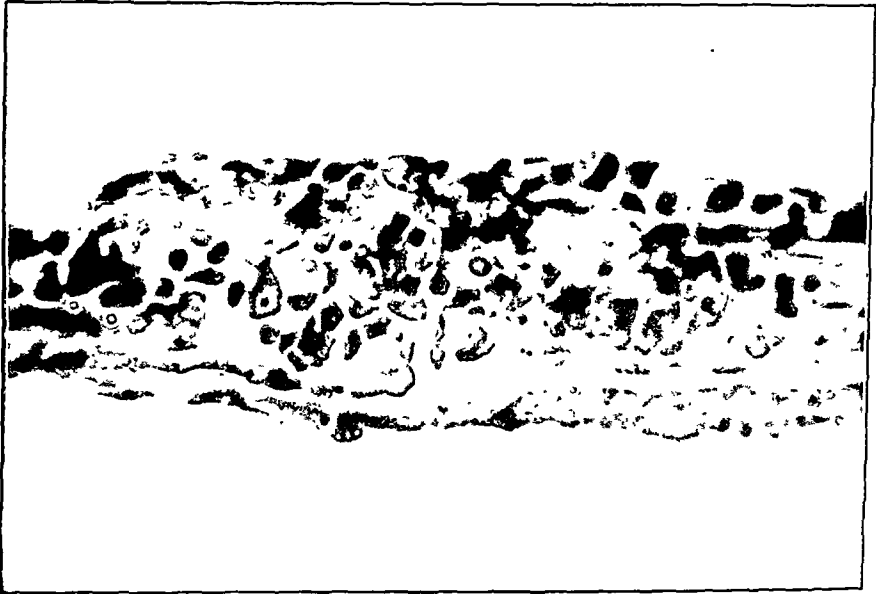
Serial passage.—On the 4th or 5th day, the inoculated egg was removed from the incubator and placed in a holder inside the glass chamber with the pointed end upwards. The upper portion was cleansed with 5 per cent carbolic acid solution and then with absolute alcohol and flamed. The top was then cut open and the contents of the eggs were poured into a sterile Petri dish. The chorio-allantoic membrane which is slightly adherent to the shell membrane was peeled off with a sterile forceps and spread out in a sterile Petri dish containing 50 per cent glycerine and examined for lesions. It was ground up with 5 c.c. of Tyrode solution and sterile glass powder in a mortar and pestle inside the glass chamber. The contents were centrifugalized and the supernatant fluid pipetted out, tested for sterility, and then used for inoculation into eggs and animals.

RESULTS.

Lesions on the chorio-allantoic membrane.—Out of the 67 eggs inoculated with different concentrations of the fixed virus, the chorio-allantoic membranes of only three eggs showed lesions. The lesions were small opaque thickenings which were discrete and slightly raised above the surface. In no case were they confluent. They never occurred regularly. Serial passage among eggs did not make their appearance constant.

Of the 34 eggs inoculated with a virulent strain of street virus, which is now under passage among fowls, none of the membranes showed any lesions.

PLATE X.



Section of a typical lesion on the chorio-allantoic membrane showing proliferation of the cells of the ectodermal layer and infiltration of these cells into the mesoderm. $\times 900$.

Microscopic appearances.—The microscopic appearances of the lesions were similar to those which have been described on the chorio-allantoic membrane of the egg after the introduction of other viruses. Membranes were fixed in Bouin's fluid and sections were stained with iron hæmatoxylin and Mann's stains. The sections showed proliferation of the cells of the ectodermal layer and infiltration of these cells into the mesoderm (*see* Plate X). The entodermal layer did not show any marked changes. Sections stained with Mann's stain were negative for Negri bodies. It is questionable whether the lesions produced can be attributed to the presence of rabies virus. Similar lesions were never seen after the introduction of sterile normal saline by the same technique nor did they appear in the majority of cases in which the virus was introduced.

Infectivity for animals.—In only two out of the ten series of experiments did suspensions prepared from the egg membranes prove to be infective to animals after the first egg passage. In neither of these were suspensions of the egg membrane infective after subsequent passage. It is interesting that none of the membranes which showed lesions was infective.

In four cases the brain of the embryo of the inoculated egg was removed aseptically, emulsified with 5 c.c. of Tyrode solution, and inoculated into rabbits. The results were negative.

DISCUSSION.

Only three out of the 101 eggs inoculated with rabies virus showed macroscopic lesions. The lesions were neither profuse, nor did they appear regularly during the subsequent passages. Egg to egg passage did not make their appearance constant.

The fact that none of the suspensions prepared from egg membranes which showed lesions indicate that the lesions were probably non-specific in character.

It was not possible to demonstrate the presence of the virus after the first egg passage. In eight out of the ten series of experiments carried out suspensions of the egg membrane were not infective after the first egg passage.

SUMMARY.

Attempts to cultivate the rabies virus on the chorio-allantoic membrane of the developing egg were unsuccessful.

ACKNOWLEDGMENT.

We wish to express our grateful thanks to Lieut.-Colonel K. R. K. Iyengar, I.M.S., Director, Pasteur Institute of Southern India, Coonoor, for his kind help and guidance during the investigation.

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CULTIVATION OF SHEEP-POX VIRUS ON THE CHORIO-ALLANTOIC MEMBRANE OF THE CHICK EMBRYO.

BY

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SHEEP-POX being a disease of economic importance, various attempts have been made to produce a satisfactory method of prophylaxis against the disease. Sacco (1809) appears to have been the first to attempt a modification of the virus. He claimed that the virus, after passage through man or calf, induced only a local reaction in sheep and also conferred immunity against the disease. Gins (1920) attempted immunizing sheep by inoculation with undiluted calf-lymph and met with success. Other workers, however, have expressed different views, with regard to the efficacy of this procedure.

Following the work of Woodruff and Goodpasture (1931) on the cultivation of bacteria-free fowl-pox virus on the chorio-allantoic membrane of the developing egg, many other viruses have been cultivated by the same technique. Attempts were made in this laboratory, on similar lines, to cultivate the virus of sheep-pox on the chorio-allantoic membrane and the properties of the cultured virus, both as regards its potentialities for prophylaxis and its virulence, as well as its antigenic relationship with vaccinia virus were investigated.

MATERIAL AND METHODS.

The experiments were initiated by using material directly obtained from a case of sheep-pox. At first, the material was used diluted and unfiltered. This caused a high mortality amongst the chick embryos and in a few, where lesions were obtained, bacterial contamination was very heavy. As a consequence of this the material was filtered. It was first ground finely in a mortar using powdered glass as an abrasive and about twice its weight of 50 per cent glycerine water was added as a diluent. The ground material was then filtered through a gradocol

membrane of average pore diameter 0.65μ . The filtrate thus obtained proved sterile and was inoculated in 0.1 c.c. amounts into 12 to 14 days-old eggs. The technique of inoculation was the same as that adopted in the cultivation of vaccinia virus in the developing egg (Rao, Pandit and Shortt, 1935). The serial passages were conducted in the same way. The lesions were cut out from the membrane, finely minced, and ground with four parts by weight of 50 per cent glycerine water, using sterile glass sand as an abrasive. About 0.1 c.c. of this ground material was inoculated into each egg for serial passages. The inoculated eggs were opened on the third day in the usual way. If opened on the fourth day greater mortality among the embryos was noticed.

MACROSCOPIC AND MICROSCOPIC APPEARANCES OF THE LESIONS.

The naked-eye appearances of the lesions produced in the egg-membrane by sheep-pox virus are quite characteristic. The whole triangular area exposed to the virus becomes a thickened yellowish-grey patch, sometimes caseating, particularly if the inoculated eggs are opened on the fourth day after inoculation. In addition, it is usual to find tiny, dot-like opacities scattered round about the lesion. There is no umbilication as found in lesions produced by vaccinia virus. Oedema of varying degrees is usually present around the lesion.

In the earlier passages, some of the lesions exhibited a hæmorrhagic tendency which, however, disappeared in the later passages.

Microscopically, the essential feature of the lesion is a considerable hypertrophy of the mesodermal layer, associated with an enormous proliferation of the ectoderm which, in some places, is caseated and sloughed off, giving a very wavy and indistinct outline on the ectodermal side. The entoderm shows only very slight hyperplasia and that only in places.

There is marked cellular invasion of both ectoderm and mesoderm chiefly by large numbers of eosinophil leucocytes, particularly in the mesoderm. Many of the ectodermal cells show oval cytoplasmic inclusion bodies which are particularly abundant on the fringe of the ectoderm.

PROPERTIES OF THE CULTURED VIRUS.

The properties of the cultured virus were investigated with regard to its infectivity to animals other than sheep, its virulence to sheep and its value in prophylaxis against sheep-pox.

EXPERIMENT ON RABBITS.

Two rabbits were shaved on the back and flanks. One was inoculated with the first three serial passage strains of sheep-pox virus separately on the right half, while the left half was inoculated with vaccine lymph and also with natural sheep-pox virus for comparison. The other rabbit was inoculated only with the third passage strain of sheep-pox virus

The results of rabbit inoculations are summarized in Table I:—

TABLE I.

Effects of rabbit inoculations with sheep-pox.

Rabbit.	Virus.	Results.
Left half ..	Vaccinia virus ..	The usual reaction. Typical take in 72 hours and scabbing on the fourth day.
	Sheep-pox virus (natural)	No take. Only a slight redness on the inoculated area, which disappeared on the third day.
1. Right half	Cultured sheep-pox virus (first passage strain).	Fairly good discrete lesions on the fourth day, and scabbing on the fifth day.
	Cultured sheep-pox virus (second passage strain).	Delayed reaction. Typical confluent lesions on the fourth day with extensive redness. Scabbing commenced on the fifth day and was complete only on the seventh day.
	Cultured sheep-pox virus (third passage strain).	Same reaction as with the second passage strain.
2. " "	Cultured sheep-pox virus (third passage strain).	Same reaction as with the second passage strain.

It will be seen that, while natural sheep-pox virus failed to take in the rabbit, the virus as modified by passage through eggs took. While the behaviour of the first passage virus is rather weak, the second and third passage viruses exhibited intense but delayed reaction.

EXPERIMENT ON CALVES.

Two calves were inoculated in the usual way each with natural sheep-pox virus and the first and third passages of the cultured virus in different dilutions. The results are shown in Table II:—

TABLE II.

Effects of sheep-pox virus on the calf.

Virus.	Dilution.	Calf No. 1.	Calf No. 2.
Natural sheep-pox virus.	Undiluted	Negative	Negative.
	1-10	"	"
	1-20	"	"

TABLE II—*concl'd.*

Virus.	Dilution.	Calf No. 1.	Calf No. 2.
Cultured virus (first passage).	Undiluted	Negative	Negative.
	1-10	"	"
	1-20	"	"
Cultured virus (third passage).	Undiluted	Confluent	Confluent.
	1-10	"	"
	1-20	"	"

The table shows that the calf is more refractory to sheep-pox than the rabbit. The first passage cultured virus failed on the calf while it gave a fair, good take on the rabbit. The third passage virus, however, behaved exactly like vaccinia virus on the calf.

EXPERIMENT ON SHEEP.

A sheep was shaved on its right flank and inoculated with the ninth passage cultured sheep-pox virus. As it was believed that the natural virus gradually lost its generalizing properties with continued passage, earlier passage material was not used, as a measure of safety. Typical pustules developed on the inoculated area on the fourth day and scabbed off on the sixth day. There was no evidence of any generalization, the mucous membranes of the mouth and nose as well as the conjunctivæ being perfectly normal. This would seem to show that serial passage in eggs modifies the character of sheep-pox virus, in that it loses its generalizing properties and becomes purely dermal in nature. This is a fact of considerable importance when its use for prophylaxis is considered.

IMMUNITY EXPERIMENTS.

With a view to ascertain whether there is cross immunity between vaccinia virus and cultured sheep-pox virus, the following experiments were carried out:—

The rabbit which was inoculated in the usual way with the third passage cultured sheep-pox virus alone (Table I), was vaccinated with vaccinia virus

in different dilutions, after a lapse of 24 days. The result is given in Table III:—

TABLE III.

Effects of vaccinia virus on rabbit inoculated with cultured sheep-pox virus.

Dilutions of vaccinia.			Result on the rabbit.
Undiluted	Good take.
1-100	Fair take—discrete vesicles.
1-250	„ „ „
1-500	Slight take—a few vesicles.
1-1,000	Very slight take— 2 „
Cultured sheep-pox virus (sixth passage).			Failure.

The results recorded show that while cultured sheep-pox virus confers no appreciable immunity against vaccinia virus in low dilutions, there is definite inhibition of the reaction in higher dilutions. The sample of vaccinia virus tested had a titre of over 1 in 10,000 on the normal rabbit. The control inoculation with cultured sheep-pox virus was a total failure which is of course to be expected.

A rabbit which has been vaccinated with vaccinia virus 20 days previously was inoculated with the sixth passage cultured sheep-pox virus. The virus was used undiluted and in dilutions of 1 in 10, 1 in 100, 1 in 200, and 1 in 400. The result was an absolute failure even with the undiluted virus, indicating that vaccinia virus confers immunity in the rabbit against sheep-pox virus (cultured). Natural sheep-pox virus was not used in the test for the obvious reason that it fails to take on the rabbit.

EXPERIMENTS WITH IMMUNE SERA.

To investigate the antigenic relationship between vaccinia virus and cultured sheep-pox virus, experiments were conducted with mixtures of immune sera and viruses. For this purpose, the anti-vaccinial serum was obtained by hyper-immunizing a calf and the anti-sheep-pox serum was obtained from a convalescent animal.

Mixtures of viruses and anti-sera were put up as follows:—

- (1) 1 c.c. of vaccinia virus + 1 c.c. of anti-sheep-pox serum.
- (2) 1 c.c. of cultured sheep-pox virus + anti-sheep-pox serum.
- (3) 1 c.c. of natural sheep-pox virus + anti-sheep-pox serum.
- (4) 1 c.c. of vaccinia virus + 1 c.c. anti-vaccinial serum.
- (5) 1 c.c. of cultured sheep-pox virus + anti-vaccinial serum.
- (6) 1 c.c. of natural sheep-pox virus + anti-vaccinial serum.

The tubes containing the mixtures were allowed to stand at room temperature for 30 minutes. Two rabbits were shaved on their backs and the shaved area divided into squares with a grease pencil. A sample of each mixture was then inoculated on a square and the rabbits were observed for one week. The results are recorded in Table IV:—

TABLE IV.

Serum-virus mixture.	Result.
Vaccinia virus + anti-sheep-pox serum	Fairly good take.
Cultured sheep-pox virus + anti-sheep-pox serum ..	About a dozen scattered vesicles.
Normal sheep-pox virus + anti-sheep-pox serum ..	Failure.
Vaccinia virus + anti-vaccinial serum	„
Cultured sheep-pox virus + anti-vaccinial serum ..	„
Normal sheep-pox virus + anti-vaccinial serum ..	„

A control rabbit inoculated with vaccinia virus, cultured sheep-pox virus and natural sheep-pox virus gave confluent takes with vaccinia and cultured sheep-pox virus and failure with natural sheep-pox virus. The mixtures with the natural virus were put in as a matter of routine and the results relating to it are of no significance as by itself it fails to take on rabbit. The anti-vaccinial serum has completely inhibited cultured sheep-pox virus in addition to vaccinia virus, and the anti-sheep-pox serum, while partially neutralizing the cultured sheep-pox virus, has, in the case of vaccinia virus, appreciably weakened the take. It is to be noted here that the results of experiments with immune sera reveal a parallelism to those obtained on immunized rabbits. The experiments are, therefore, reciprocally confirmatory.

INFECTION OF SHEEP IMMUNIZED WITH CULTURED VIRUS WITH THE NATURAL VIRUS.

The sheep which had been vaccinated with the cultured virus on its right flank, the procedure resulting in only a local reaction, was, after a lapse of one month, inoculated on its left flank with the natural virus. The result was only 2 vesicles on the inoculated area. There was no generalization of the virus, as evidenced by healthy mucous membranes of the mouth, nose, and conjunctivæ. A normal sheep inoculated at the same time with the same natural virus showed a rather mild generalized infection with fairly intense local reaction, a few vesicles on the mucous membranes of the nose and mouth but no general eruption over the whole body and no constitutional symptoms. This was probably due to ageing of the natural virus but the

net result helps to bring out the potentialities of the cultured virus for prophylaxis.

DISCUSSION.

Sheep-pox is a disease regarding which there is much conflict of opinion as to its pathogenicity to animals besides sheep and its immunological relationship to other pox viruses, particularly vaccinia.

It is not improbable that this divergence of opinion may be due to differences in virulence of the materials used by different workers.

The present study confirms the observation that natural sheep-pox material fails to infect on direct transference to both the calf and rabbit. However, even the first passage cultured virus took fairly well on the rabbit. The subsequent passages showed a distinct change with an enhanced virulence and delayed reaction. The cultured virus was also successfully transmitted to the calf, though at a later passage (third). This alteration of the virus was also characterized by a striking decrease of virulence for the sheep, the reaction being a mild and purely local one without any sign of generalization. This result may be compared with that obtained by Sacco (*loc. cit.*), who observed that sheep-pox, as a result of passage through man or calf, lost its virulence for sheep and only caused a local reaction at the site of inoculation.

It is a moot point as to whether sheep-pox virus is converted into vaccinia by such extra-ovine passages. There is a section of opinion that it is so converted and Blaxall (1930) believes that the balance of evidence establishes the contention that 'sheep-pox can be transformed into vaccinia by passage through the goat, calf, or rabbit'.

On the other hand, Findlay (1936) observes that it is a well-known fact that rabbits and calves become spontaneously infected with vaccinia in lymph establishments, that, where claims of conversions of sheep-pox into vaccinia have been made, precautions against accidental contaminations have been inadequate, and that, before such claims can be accepted, more extensive and carefully-controlled experimental evidence is needed.

The present work, based on the passage of sheep-pox virus through eggs, offers no evidence of the conversion of the virus into vaccinia. This statement is, however, subject to the qualification that the total number of passages conducted was small. The question as to whether, as a result of further passages, the virus would finally change into vaccinia is left unsettled. That the virus is altered in character as a result of such passages is clear from the experiments on rabbits, calves, and sheep. The experiments in immunized rabbits and with immune sera clearly indicate that vaccinia virus and cultured sheep-pox virus do possess partial common antigenicity.

As regards the question of prophylaxis against sheep-pox, opinion is again sharply divided on the utility of vaccinia virus, as an immunizing agent. Bridre (1931) attributes positive results to the fact that the experiments were conducted in lymph establishments.

In the present investigation, the potentialities of the cultured virus as a prophylactic agent are indicated, but as the observations have been based on a single

experiment owing to the loss of the cultured virus, they must be accepted with some reserve.

SUMMARY.

1. The cultivation of sheep-pox virus on the chorio-allantoic membrane of the developing egg has been described and the naked-eye appearances and histological features of the lesions are defined.

2. The cultured virus is definitely modified in character by passage.

3. Immunity experiments indicate common antigenicity to a limited extent between the cultured virus and vaccinia virus.

4. Cultured virus, while inducing a purely local reaction in sheep, appreciably protects against subsequent infection with the natural virus. The possibility of its use for prophylaxis is suggested.

ACKNOWLEDGMENTS.

My thanks are due to Lieut.-Colonel H. E. Shortt, I.M.S., Director, King Institute, for kind permission to publish these observations, and to Rao Sahib M. Ananthanarayana Rao of the Madras Veterinary College and Dr. Achar of the Mysore Veterinary Institute for supplying the sheep-pox virus and antisera.

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QUANTITATIVE STUDIES ON THE BIOLOGY OF *XENOPSYLLA CHEOPIS* (SIPHONAPTERA).

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INTRODUCTION.

It is established that in most parts of the world plague is transmitted by *Xenopsylla cheopis*, and that there is a considerable degree of correlation between outbreaks of the disease and the abundance and the local distribution of the insect. But our knowledge of the flea's biology, particularly of the causes of its multiplication or decrease, is still incomplete, and what we know is based mainly on the flea count, which is an established and valuable method, though we can hardly think that it gives a good measure of a natural population of fleas. Apart from flea counts one must remember that there is a large amount of relevant fact accumulated by laboratory workers, most of it within recent years. They have, for instance, provided a considerable amount of knowledge about the length of life of fleas under different conditions of temperature and humidity and about the thermal death point of different stages of the insect. Most of this work has been carried out under physiological rather than natural conditions and only for short periods of time. These laboratory studies are excellent as a foundation, for they define some of the insect's limits in climate and they give information about the site of loss of water. After setting out his own experimental results the author hopes to discuss the subject at greater length.

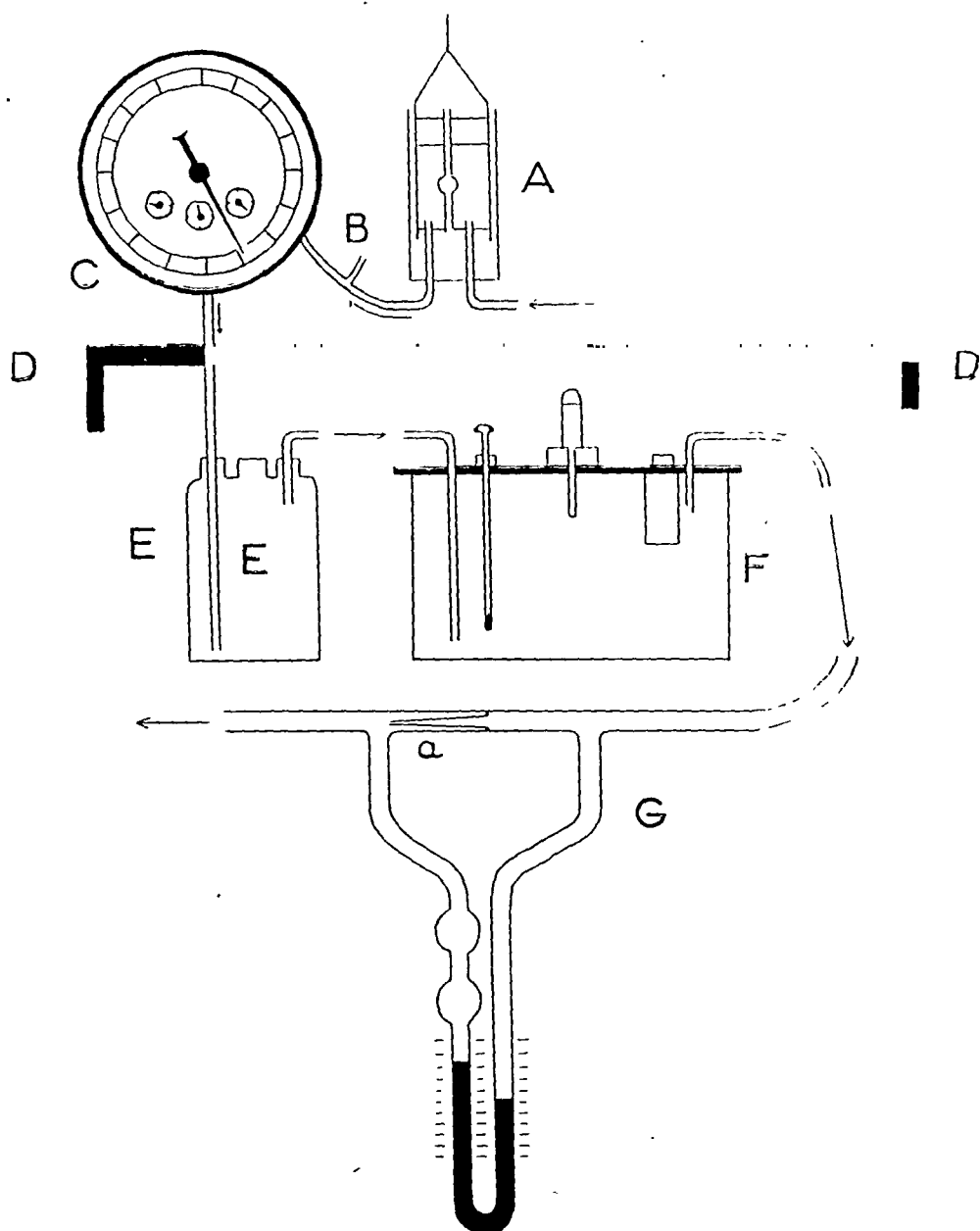
It should now be possible to devise more elaborate laboratory experiments to give quantitative information about the climatic factors which influence the multiplication of these insects. For this we need a synthetic mouse-hole in which to maintain a rodent and fleas for a relatively long period under defined environmental conditions. It is clearly desirable that the conditions should be nearly natural, though at the same time they must be capable of being measured and controlled. One must, moreover, be able to enumerate both the original parent fleas and their offspring. No such work has been attempted so far as is known, partly perhaps because the necessary technical methods are not widely known. The author has not been completely successful in carrying out this rather ambitious

programme, but with the apparatus which has been gradually evolved some success has been achieved. The account of the technique is detailed, because it seems that with this apparatus a great amount of quite new work might be carried out. Moreover, with slight modifications the apparatus might be made suitable for other hosts and other problems.

METHOD.

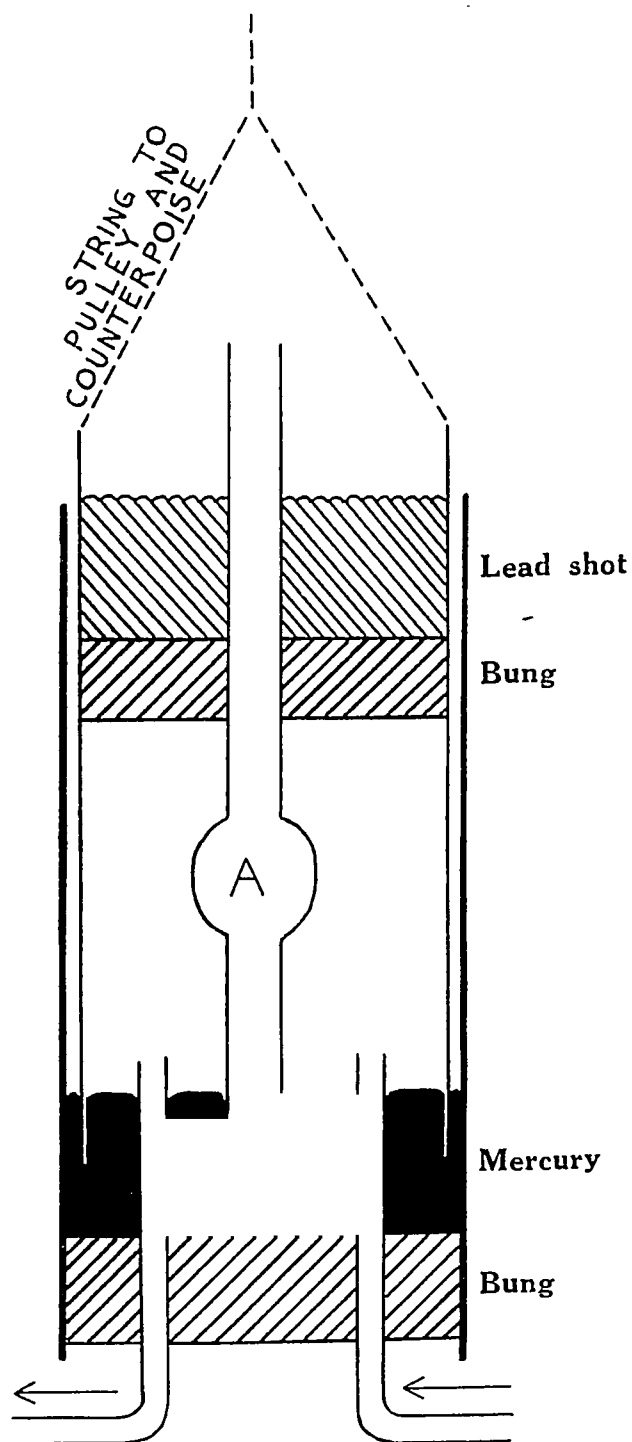
General scheme.—The purpose of the experiment is to discover the climatic conditions under which fleas live and multiply most easily. It is proposed that a rodent and a small population of fleas should be kept, for at least a week, in air of which the temperature and humidity are controlled. The mouse produces so much water and carbon dioxide, particularly at temperatures below 30°C., that it is impossible to regulate the humidity in still air in a closed chamber. For this reason the apparatus used by Stoughton (1930), Carrier cabinets, and other enclosed containers are not suitable. For the work in view we must work in a steady stream of air, which is brought to the required temperature and humidity before it enters the chamber in which the mouse and fleas are kept. One may, therefore, say that the apparatus consists of two principal parts: that for regulating the volume, temperature, and humidity of the air, and that in which the rodent and fleas live and in which the experiment itself is actually performed. The general plan of the apparatus will be evident from Text-fig. 1. If it is to work well for long periods careful attention to details is necessary.

The air supply.—The compressed air supply is available day and night, but fluctuates in pressure between 15 lb. and 30 lb. above that of the atmosphere; the fluctuation is somewhat irregular. It is clear that for these experiments a very steady flow of air is required, for if the pressure and therefore the flow of air falls, water produced by the mouse will accumulate in the experimental chamber and raise the humidity in it. With the help of Mr. T. C. Angus a valve has been devised which allows some air to leak away and passes a very steady flow (Text-fig. 2). When this valve is connected to the compressed air supply the inner part of it floats on the mercury, allowing some air to escape up tube A; the rest of the air passes on to the meter and apparatus. Tube A must be rather wide (6 mm. to 7 mm.), otherwise small pellets of mercury will occasionally be blown through it; the bottom of A is cut off obliquely, which causes it to float on the mercury without bumping. This valve is delicate, and it is possible, by altering the amount of lead shot, to adjust it so that the rate of flow through a particular apparatus is fixed within about 0.5 litre per hour. Needless to say, any alteration in the humidifying part of the apparatus will alter the resistance to the flow of air through the whole; the volume of air passing must then be brought back to the required figure by adjusting the weight on the valve. This valve has proved extremely satisfactory in practice and runs for many weeks continuously without attention. From it the air passes to the meter (C, Text-fig. 1) which continually records the flow. The meter, made by Messrs. Siebe Gorman, is of the type used by mammalian physiologists. The air, of which the flow is controlled and measured, then enters a large incubator set at the temperature required for the experiment. In Text-fig. 1 the heavy line DD indicates the wall of the incubator; all parts of the apparatus below that line are to be understood to be inside the incubator,



TEXT-FIG. 1.

General lay-out of apparatus, not to scale. A, B, and C are on the laboratory bench, DD is the wall of the incubator, and the rest of the apparatus is inside it. A, valve, regulating flow of air to meter C and rest of apparatus. E, Woulff's bottle (one of several in series), to bring air stream to required humidity. F, experimental chamber (see Text-fig. 3). G, Venturi meter.



TEXT-FIG. 2.

Valve which allows a very steady stream of air to pass on to meter and apparatus. Air enters and leaves as shown by arrows. Any excess above the determined quantity escapes by tube A. Diameter of valve about 7 cm. to 8 cm.

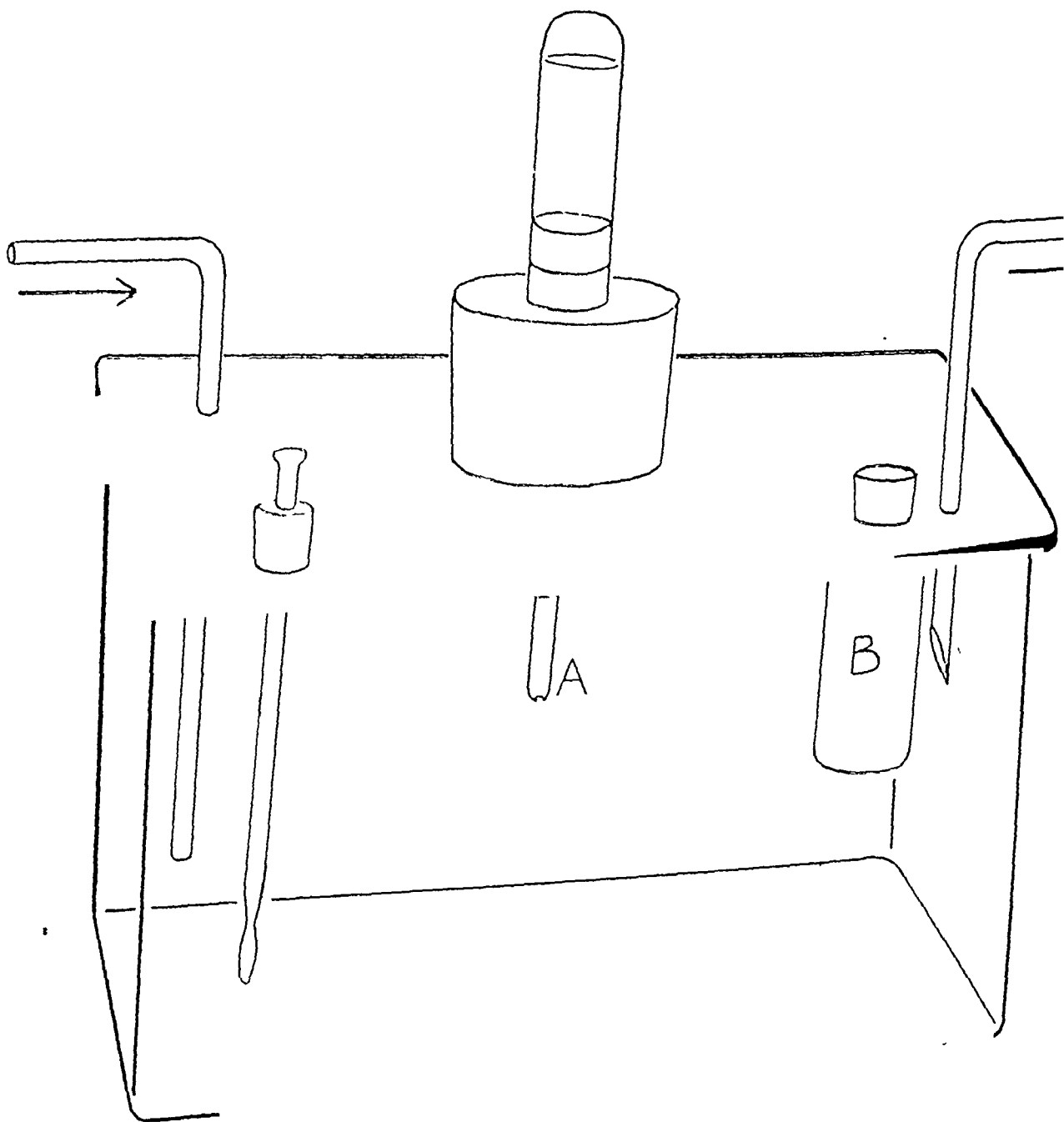
In the incubator the stream of air is brought to the required humidity by contact with mixtures of potassium hydroxide (or sulphuric acid) and water. There is no doubt that this would be most quickly done if the air were passed in a finely divided stream through jets or sintered glass. The objection to doing this is that it would require a pressure of many centimetres of mercury, which would tend to produce leaks. It has, therefore, seemed better to circulate the air over a large surface of pumice contained in a series of wash-bottles, the pumice being wet with potassium hydroxide (or sulphuric acid), but not immersed in liquid. One such wash-bottle is shown in Text-fig. 1, E. but it is to be understood that four are generally used in series. They offer very little resistance to the flow of air, and when the whole apparatus is assembled the pressure at the point B in Text-fig. 1 is only about 2 mm. above that of the atmosphere. By this method the relative humidity of the air entering the experimental chamber can easily be brought to within about 2 per cent of the required figure and maintained there for as long as may be desired. It is also found that the air has reached the temperature of the incubator by the time it has been brought to the required humidity. The incubator contains so much apparatus that it is desirable to stir the air in it, by a small fan, wired in series with the heating elements. The temperature in the incubator is recorded by thermograph. The particular incubator which was used had glass doors. Direct sunlight never entered the incubator, but there was a dim light in it during hours of daylight. It would be well in future to work in darkness, thus eliminating one possible variable.

It will be seen that the apparatus described will supply and measure a certain volume of air per hour, having brought it to the required temperature and humidity. This air is then passed into the experimental chamber, which is also in the incubator.

The chamber.—The chamber (Text-fig. 1, F. and Text-fig. 3), in which the rodent and fleas are to live together for a period of days or weeks, consists of a glass battery jar, 7.5 inches \times 5.5 inches deep (say 18 cm. \times 12.5 cm. \times 13 cm.). The edges of the glass are ground to make a satisfactory joint with the top, which is of copper sufficiently thick (about 3 mm.) to ensure that it does not bend. When the apparatus is being assembled modelling wax is spread evenly along the top of the glass, the metal top is heated and pressed evenly upon the wax. Metal piping, passing through the copper lid, provides inlets and outlets for air at opposite corners; there is also a hole in the lid to carry a large rubber bung through which the mouse is supplied with food and water. Holes are also made on the top for carrying a thermometer, passed through a rubber bung, and for inserting weighing hygrometers (Buxton and Mellanby, 1934). Text-fig. 3, B, is a cylinder of perforated metal which protects the weighing hygrometer from the mouse. A hook on the underside of the lid (not shown in the figure) may carry a watch hygrometer, but this was not used in the later experiments, in which the humidity was measured by an absorption method (*see below*).

The recording meter (Text-fig. 1, C) shows that the correct volume of air has been passed into the apparatus, but there are many points at which leaks might occur, and if the flow of air through the chamber itself is less than it should be, water derived from the mouse will accumulate and raise the humidity. One therefore needs to be assured that the correct volume of air has not only entered the

apparatus, but passed through it. This is shown by a Venturi meter (Text-fig. 1, G) attached to the outlet from the chamber. The internal nozzle *a* sealed inside the horizontal limb raises the pressure in the right limb of the manometer, and depresses the oil to a level which is determined by the rate of flow of the air. By



TEXT-FIG. 3.
Experimental chamber, through which conditioned air is passed. A, tube from which mouse drinks. B, cylinder of perforated metal containing a weighing hygrometer.

glancing at the Venturi meter, which is visible through the glass door of the incubator, one may assure oneself at any moment that the apparatus is working correctly.

Test of apparatus.—It will be remembered that the apparatus delivers a known volume of air of controlled temperature and humidity to the chamber in which the rodent lives with its supply of food and water and a population of fleas. After the air has entered this chamber containing the mouse, etc., its humidity may be disturbed in several ways. There must be some evaporation from the surface of the mouse's water-supply, but this may be disregarded in comparison with other sources of error (*see* below). There may also be a small error owing to the fact that the mouse's daily ration of food is not in humidity equilibrium with the air. But by far the greatest disturbance of humidity must be that due to water coming from the lungs and kidneys of the mouse. A few approximate measurements carried out in a stream of dry air show that under those circumstances a mouse may give out at least 200 mg. of water per hour in dry air at 20°C. This is likely to be close to the mouse's largest output, for at higher humidities evaporation from the lungs will be less, and at higher temperatures the mouse's metabolic rate and, therefore, its output of water will be lower. The great effect of temperature on the mouse's output of water is apparent from Table III. At 24°C. the mouse will raise 30 litres of air per hour from 60 to 70 per cent to 80 to 90 per cent but at 32°C. the rise is about 2 per cent. These figures correspond to an output of 130 mg. of water, under the above conditions, at 24°C., and about 20 mg. at 32°C. It is clear that the humidity of the air leaving the chamber must always be higher than that at the point of entry, though the increase of water will be regular provided the air continues to flow at the same rate.

It would be insufficient to measure the humidity in the outgoing air alone, if there were pockets of damper air in particular parts of the apparatus. This point was investigated, making use of a special chamber with four additional holes drilled in the roof; these were closed with rubber bungs carrying weighing hygrometers suspended inside the cage, protected from the mouse by perforated metal (as Text-fig. 3, B). The weighing hygrometer which was used was a filter-paper loosely folded and previously calibrated over a range of humidity. When it was subsequently exposed to conditions in the chamber and weighed, the humidity at particular points in the chamber could be deduced from its weight. Passing 30 litres of air per hour through the apparatus, which contained the mouse together with its bedding, etc., it was found that four places near the top of the cage gave humidities generally within 2 per cent, always within 4 per cent of one another. Close above the bedding the humidity was rather more irregular, perhaps owing to the presence of the mouse, but even here the extreme range of humidity was 8 per cent or less. There was also a tendency for the humidity to be greater at the bottom than at the top, generally by 4 per cent or 5 per cent. These figures were obtained at 28°C.; owing to the effect of temperature on the mouse's output of water one would expect that the uniformity of humidity in different parts of the apparatus would be greater at high, less at lower temperatures. In view of the fact that the fleas themselves, and their progeny, live in the bedding, it would clearly be interesting to obtain facts about the humidity of the air spaces in it. I attempted to get this information by determining the water content of bedding in different parts of the cage. But the material is so heterogeneous, consisting

of original bedding, fresh dung, fragments of food, etc., that the proportion of water in it varies erratically. It seems probable that the humidity in the bedding is very near to that of the air circulating over it, for the material is loose and only about 2 cm. deep.

It seems then that with an air flow of 30 litres per hour turbulence is sufficient to give a nearly equal humidity in all parts of the apparatus. It may also be stated that local differences in humidity within the chamber are always less than the difference in humidity between the incoming and outgoing air. It must be remembered that the weighing hygrometer, though very sensitive, does not react very quickly; those used came to equilibrium in still air in three hours but not in one. They would not, therefore, be capable of detecting momentary differences in humidity such as might be caused by the breath or urine of the mouse, but such momentary differences in particular parts of the chamber have no significance in an experiment which runs for several weeks.

It has, therefore, been established that, provided the air flow is steady, the control of humidity is good, and that there are no major differences in humidity in different parts of the chamber. That being known, we confined ourselves to measuring humidity once a day, first in the outgoing and then in the incoming air; humidity was measured by passing 5 litres of air through Landsiedle bulbs containing concentrated sulphuric acid and weighing the water which was absorbed. At the same time one read the recording air meter, observed the thermograph in the incubator, and supplied the mouse with its food and water by removing the large rubber bung. The Venturi meter, which was visible through the glass door of the incubator, was observed from time to time as a check on the air flow.

It is probable that the rodent's production of carbon dioxide is also a factor to remember in designing such experiments as these. One may calculate approximately the output of CO_2 making use of the generally accepted figure that a mouse's production of heat is 212 calories per kilo per 24 hours. This figure is equivalent to 44 c.c. CO_2 per hour, for a mouse of 25 g. With an air flow of 30 litres per hour the concentration of CO_2 would be 0.15 per cent which probably has no significant effect on the fleas. But if one were using several mice, or a lesser air flow, the increase in CO_2 would become important.

The apparatus as here described gives extremely satisfactory results, except that when the air is close to saturation the bedding becomes wet, foul and mouldy, and no fleas of the F generation emerge. Moulds are occasionally troublesome even when the outgoing humidity is as low as 80 per cent.

The rodent.

When the apparatus was ready for use a layer of bedding half an inch deep was put into the chamber. For bedding we took material from a jar in which mice and fleas had previously lived, and added a pinch of dried blood to it. We may, therefore, assume that the bedding contained substances necessary for the development of flea larvæ (Sharif, 1937a). The food for the mouse consisted of whole oats 50 per cent, oatmeal 46 per cent, wheat bran $2\frac{1}{2}$ per cent, yeastrel (dry weight) 1 per cent, cod-liver oil 0.5 per cent: the food was ample in quantity. This diet is similar to Watson's diet N_2 (Watson, 1937) on which it is found that mice live well, but a large proportion of oats has been added because the

husks of partly eaten grains appear to be favourable to the early stages of the flea. The mouse was given water to drink, which it obtained by sucking from the bottom of the tube shown in Text-fig. 3, A; the surface of water exposed inside the chamber is only about 3 mm. in diameter, so that evaporation from it is not likely to have any measurable effect on the humidity inside the chamber. The food and bedding were autoclaved in order as far as possible to avoid trouble from moulds and mites.

Mice of different weights add very different quantities of water to the environment: in order to obtain reproducible conditions, it was necessary to use a single mouse weighing about 20 grammes. No evidence was found that the health of the mouse was affected by the different conditions of temperature or of humidity to which it was subjected. A possibility exists that, inasmuch as mercury had to be used in the valve, traces of mercury vapour sufficient to cause poisoning may have been carried into the chamber. There is no evidence that the mice or fleas were affected by mercury vapour, but this source of trouble is so subtle that the possibility of its existence should be recorded. It seems probable that one could avoid using mercury by making a similar valve in which the moving part was celluloid floated on water (or on medicinal paraffin).

The fleas.

All the experiments here described have been carried out with the tropical rat flea, *Xenopsylla cheopis*, our stock of which was brought from Ceylon to London by Dr. L. F. Hirst in 1930. Since that date we have continued to breed the insect easily and in great numbers by the method described by Leeson (1932*a*). The method is ingenious, making use of semi-automatic devices and giving relatively little trouble to the staff. It ensures a large supply of fleas and makes it easy to obtain insects of a particular age, either fed or not.

The experiment.

After the mouse has been put in the apparatus everything is allowed to run for two or three days, in order that the bedding may come into equilibrium with the air stream. When the humidity of the outgoing air has become steady we take unfed fleas less than one day old, selecting an equal number of males and females, generally 15 to 50 of each sex. The day on which this is done is referred to as day 0. These fleas, called P (parental) fleas, are inserted into the chamber and left undisturbed with the mouse for the duration of the experiment, generally 7 days, but occasionally as long as 18 days. At the end of that time the chamber is removed from the incubator, and the fleas in the bedding counted; the mouse is lightly anaesthetized and the fleas on it removed and counted, the sex of the fleas on the mouse and in the bedding being recorded. Owing to the short duration of the experiment one is quite confident that all fleas recovered belong to the P generation.

In the experiments here described the material from the cage, consisting of the mouse's droppings, unconsumed food and original bedding, is again incubated at the temperature of the experiment and at the humidity which had prevailed inside the chamber; for instance, if the incoming air had had a relative humidity of 60 per cent and the outgoing of 70 per cent the bedding was incubated at 65 per cent. Every day it was tipped into a large battery jar, searched for fleas and the number emerging (of the F or filial generation) recorded.

Work of the future.

It is desirable that work of this nature should be carried out with rats because they are the principal natural hosts of *X. cheopis*. Before this can be put in hand approximate figures will be required on the amount of water and CO₂ given off by the rat, and it will be necessary to use a larger experimental chamber.

It would also be interesting to extend the period of the experiments to cover the whole life of the P fleas. Once a week the P fleas might be counted and the bedding removed and incubated for progeny (F fleas).

If the experiments are to help to explain the biology of the flea in nature it is particularly important that work should be done at very high humidities, for there is some evidence (Buxton, 1932, 1936) that even in dry places and seasons the atmosphere in a rat-hole, or in spaces in the soil, is nearly saturated with moisture. Work at high humidities (over 90 per cent) is at present impossible in my apparatus, for the bedding becomes moist and mouldy, and no F fleas are produced. One may suppose that in nature this would not occur, for much of the liquid water would pass away by capillarity from the rat's nest into the soil. It might be possible to imitate natural conditions, covering the bottom of the experimental chamber with plaster of paris which had set and become thoroughly dry.

Table I shows inconsistencies in the proportion of adult fleas surviving even for short periods such as seven days: this may be overcome in future by the use of baby rodents (see Postscript).

EXPERIMENTAL RESULTS.

Two selected experiments.

The results obtained with this somewhat elaborate apparatus may best be understood by selecting two experiments (N and O), and seeing what may be learnt from them. In each of these the recording meter showed that the air flow had been maintained very close to 30 litres an hour, and records of the water content of the air entering and leaving the chamber showed that the regulation of humidity had been good: in general the two experiments may be regarded as successful. The temperature was 28°C. in each. The experiments differed from one another in the following particulars:—

				Experiment N.	Experiment O.
Humidity, ingoing		56 to 62 per cent	67 to 69 per cent.
„ outgoing		64 to 72 „	89 to 93 „
Period		9 days	7 days.
Number of <i>cheopis</i> (P) at start	..			30 (15♂, 15♀)	40 (20♂, 20♀).
„ „ „ „ at finish	..			23 (12♂, 11♀)	34 (18♂, 16♀).
F fleas produced		106	209

It appears that the increase in relative humidity of the air in passing through the chamber was about 10 per cent in experiment *N* and over 20 per cent in *O*: this may perhaps be put down to differences in activity of the two mice. We cannot at present say whether the difference is important or not.

Taking the figures for *P* fleas (males and females together) one sees that in *N* the population fell from 30 to 23, in *O* from 40 to 34, the period of the experiments being 9 and 7 days. In order to obtain comparable figures one may calculate what number of fleas was alive in experiment *N* on the seventh day, assuming that the decrease was regular and on a geometrical progression. The necessary calculation is as follows: x and y are the number of fleas in an experiment at the beginning and end of the period respectively: r is the constant factor in the geometrical progression and gives the rate of loss (or gain) of population; n is the number of days. Then, on our assumption that the decrease is regular and geometric,

$$y = xr^n$$

$$\text{i.e., } \log y = \log x + n \log r$$

$$\therefore \log r = \frac{\log y - \log x}{n}$$

$$\therefore \log r \text{ (in experiment } N) = \frac{1.362 - 1.477}{9} = \frac{-0.115}{9} = -0.013$$

We want to know the population (y_7) on day 7. Going back to the original formula in its log form we may say that

$$\log y_7 = \log x + (7 \times -0.013)$$

$$= 1.477 - 0.091$$

$$= 1.386$$

$\therefore y_7 = 24.3$, which is the number of fleas alive on day 7, according to our assumption.

The number of fleas (observed or calculated) alive on day 7 is 24.3 in experiment *N*, 34 in experiment *O*. These figures are 81 per cent and 85 per cent of the original populations, so that in this respect the agreement between the two experiments appears to be satisfactory.

Taking sexes of fleas separately, the actual number of survivors on the last day of the experiment was:—

Experiment <i>N</i>	12 ♂	11 ♀	total 23
„ <i>O</i>	18 ♂	16 ♀	„ 34

The figures indicate that there is little difference in survival between males and females. In experiment *O* the fleas were distributed as follows between the mouse and the bedding at the end of the experiment:—

♂ ♂,	on mouse 1,	in bedding 17
♀ ♀,	„ „ 0 „ „	16

No similar figures are available from experiment *N*, in which the mouse died a few hours before the conclusion of the experiment.

Let us now consider the offspring (F or filial fleas) produced in the two experiments. The gross figures are 106 in experiment *N*, 209 in experiment *O*; but there are differences in the original number of females, in their percentage survival, and in the duration of the experiments, so that the crude figures cannot be used in comparing the number of offspring in the two experiments. One may, however, get comparable figures by calculating the number of females surviving each day, using the formula given above. The following results are obtained:—

Experi- ment.	Day :—0	1	2	3	4	5	6	7	8	9	Total ♀ days excluding days 0 and 1.
<i>N</i> ..	15	14.5	14.0	13.5	13.0	12.6	12.2	11.8	11.4	11.0	99.5
<i>O</i> ..	20	19.4	18.8	18.2	17.6	17.0	16.5	16.0	104.1

One may then add the number of female days in each experiment, excluding days 0 and 1, on which we have reason to think that the female does not lay eggs. This puts us in a position to compare the productivity of the two experiments, which is as follows:—

Experiment.	♀ days.	Offspring (F).	F per ♀ day.
<i>N</i> ..	99.5	106	1.06
<i>O</i> ..	104.1	209	2.00

The experiments also give approximate information about the length of time which elapses from the laying of the egg to the emergence of the adult flea: the figures are calculated in days from the start of the experiment. In interpreting the figures it should not be forgotten that the parent fleas are thought to have laid no eggs on days 0 and 1. In bedding derived from experiment *O* the first flea of the F generation emerged on day 23, the last on day 44. Within those extremes emergence was very evenly spread over a long period (particularly when we remember that experiment *O* lasted only 7 days, and that egg laying occurred only on the last 5 of them). The spread of emergence is probably due in part to females emerging many days before males (Leeson, 1932*b*; Hirst, 1926). If a record of sexes of F fleas had been kept one could work out the mean duration of the early stages for males and females separately. But, as the sexes were not recorded, it would be misleading to present a mean. It is probably better, therefore, to quote no mean or mode, and merely to record that 80 per cent of emergences took place from day 24 to day 34 inclusive. Similarly, in experiment *N*, the first and last emergences were on days 27 and 40, 80 per cent of them between days 29 to 38.

Experiments *N* and *O* have been worked out rather fully, so as to make it easier to study the whole body of fact which has been gathered together.

Survival of P fleas.

Let us consider more briefly the results of all experiments, of which there are 27, including the two which have just been described in detail: but five of these experiments will be disregarded, for a reason set out later. The total number of experiments is small, and the conclusions can only be tentative.

Consider first the figures for survival of fleas of both sexes of the P generation. In order to investigate the effect of temperature and humidity upon the survival of the fleas one must standardize the figures, which has been done by calculating the number which would have been alive on the 7th day for those experiments which lasted for longer or shorter periods. The available information is set out in Table I, which includes not only the experiments in which the regulation of humidity was satisfactory, but also a number of others, most of them carried out before the mercury valve had been devised, in which the relative humidity fluctuated as much as 20 per cent: these experiments are shown in italics in the table. It is evident that there are several completely unsatisfactory experiments, and we are probably justified in excluding those five shown in brackets, in which the proportion of survivals on the 7th day was less than 50 per cent. This high mortality is probably due to the mouse eating a large number of fleas (*see* Postscript). The remaining 22 experiments appear to indicate that the percentage of P fleas surviving to the 7th day is not influenced by temperature or humidity within the range studied. Under the conditions which prevailed in my experiments it is probably justifiable to assume that the fleas fed sufficiently to restore any loss of water which low humidity had caused. In this respect they probably behaved like *Cimex* which, as Mellanby (1935) has shown, can suffer a great loss of water, replacing it at the next feed. For this reason *Cimex* can be bred successfully at almost any humidity provided that it is fed frequently.

TABLE I.

Showing the percentage of fleas, of both sexes taken together, which survived on day 7. Some percentage survivals are observed, others calculated. The figures give survivals in individual experiments.

Humidity.		Temperature, °C.		
Mean per cent ..		24°-25°	28°	32°
Under 30	78. 70.	..
31-50 ..	61.		68.	..
51-70 ..	91. 62. (44) (30)		92. 85. 81. 80. 78.	73
71-90 ..	90. 68. 66. 64. (28)		(25) (0).	78. 78
91 and up ..	77. 70		79. 79	..

It is reasonable to inquire whether the number disappearing is greater in experiments in which many fleas were used than in those with less, as it might

tend to be if destruction of fleas by the mouse is a common event. The experiments have been grouped according to the total number of fleas put in at the start; the survivals are those observed or calculated for the 7th day; the figures, which are as follows,

Original number of fleas.	Number of experiments.	SURVIVALS, PER CENT.	
		Mean.	Range.
122 ..	3	75.5	68-79
70-50 ..	10	74.8	62-92
40-30 ..	9	77.2	61-90

give no indication that on the average the original density of the flea population affects the proportion surviving.

Leeson's (1936) experience was evidently not dissimilar to my own. He kept *X. cheopis* with a mouse in a jar at room temperature. In a total of 19 experiments, he started with 1,814 fleas, only 66 per cent of which survived to the 7th day. In individual experiments the recovery rate ranged from 12 to 88 per cent.

The amount of information about the survival of the male and female *P* fleas is limited, as the sex of survivors was not recorded in the earlier experiments. Figures from 9 of the experiments give no indication that mortality is differential over the period of the experiments (6 to 18 days). The results of experiments *N* and *O* quoted above are a fair sample of the whole.

Position of surviving P fleas.

It is generally assumed in practical studies in the field that the proportion of fleas found on the rats bears some relatively constant relation to the population of wild fleas: the use of the 'flea count' is based on that assumption. A record was therefore kept of the number of fleas which were found on the mouse's body or in the bedding at the time when the experiment was brought to an end. The available figures from the 22 experiments are grouped by temperature in Table II; the information is incomplete because the sex of survivors was not recorded in certain early experiments. The table shows that there is a considerable variation in the proportion of fleas found on the mouse at the end of an experiment and that this proportion is not related to temperature. In groups of experiments at different temperatures the percentage of fleas on the mouse ranged from 4 to 28: taking all experiments together, the proportion was 12.8 per cent. I also grouped the original figures according to the humidity, but found no relation between the proportion of fleas on the mouse and the humidity. It would perhaps have been supposed that at a lower humidity the insects would take blood more frequently and would therefore be more frequently found on the mouse, but there is no evidence that this is so.

It is evident from Table II that (in the two groups of experiments in which the figures were preserved) the number of female fleas on the mouse is much greater than the number of males, approximately equal numbers of males and females being alive in the chamber at the time the examination was made. Applying the test for goodness of fit to the results obtained at 32°C. one finds that the disproportion between males and females is highly significant ($P < 0.01$).

TABLE II.

Showing the total number of fleas which survived, and the number found on the mouse: also the numbers of the sexes in all survivors and in those on the mouse.

Temperature, °C.	Number of experiments.	NUMBER OF SURVIVING P FLEAS.			FLEAS ON MOUSE.			Per cent on mouse.
		Total.	♂	♀	Total.	♂	♀	
24	5	152	78	74	6	0	6	4
25	6	93	?	?	24	?	?	26
28	8	351	?	?	31	?	?	9
32	3	100	46	54	28	7	21	28
TOTAL ..		696	89	12.8

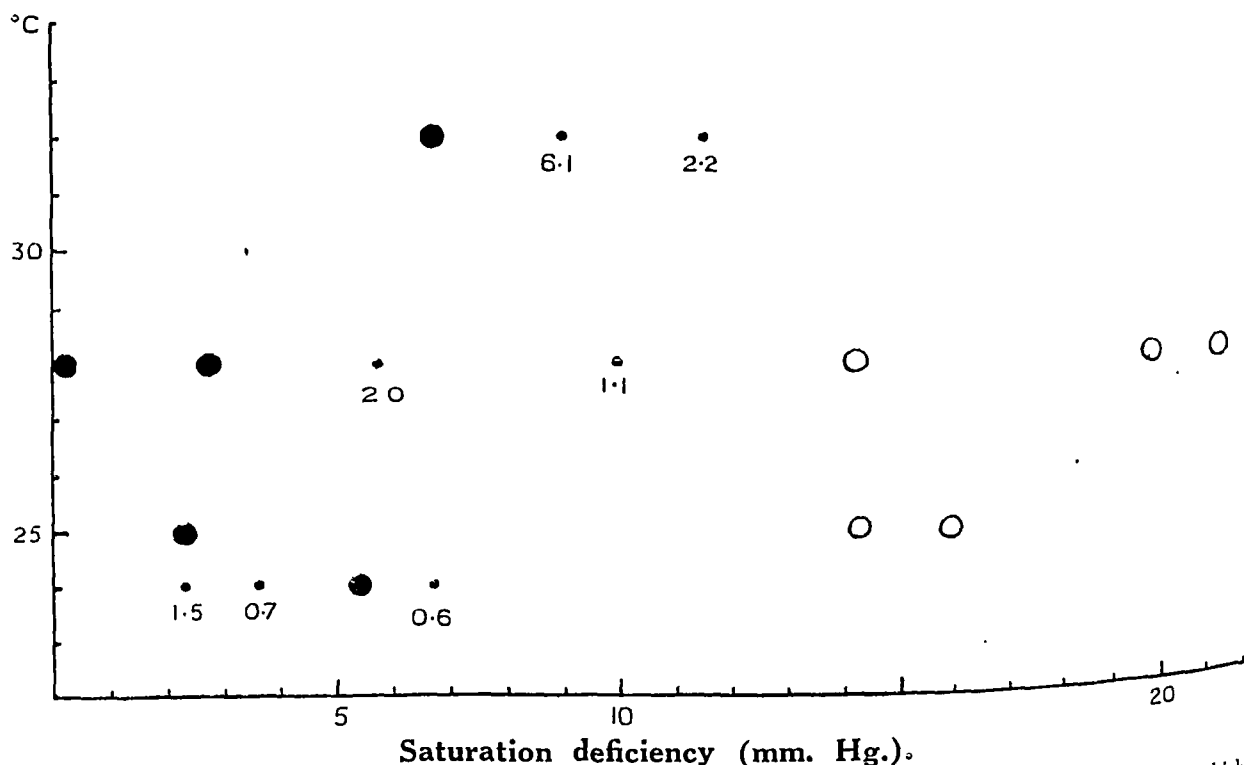
Production of F fleas.

In each experiment the bedding was taken at the time when the fleas were counted, incubated at the temperature and mean humidity which had prevailed during the experiment, and examined daily for emerging fleas of the next (F) generation. The method of working out the figures so as to discover the number of offspring (F fleas) per ♀ (P) day has already been explained. As the method entails a considerable amount of work it was carried out only on seven experiments (including N and O), in which the control of the air flow, and therefore of the humidity, was good. The results obtained from these seven experiments are shown in Table III. At first sight it is not easy to interpret the table or explain the considerable differences in the production of F fleas, but it can be seen that at each temperature production was greater at the higher humidity. As this occurred at each of three temperatures, it appears probable that the precise degree of humidity has much influence on the production of fleas. The table also makes it clear that, as would have been expected, the number of offspring per day is greater at higher temperatures. Whether the total production per female is affected by temperature is not known.

In the earlier part of this paper it was found that humidity had little or no effect on the P fleas; it was, therefore, indifferent whether it was expressed as

relative humidity, which is in some ways more convenient, or as saturation deficiency; but in considering the F fleas it is evident that humidity is an important factor, presumably because of its effect upon the larvæ. As it has been established that for biological purposes saturation deficiency is the better measure of humidity, I have graphed the data given in Table III against temperature and saturation deficiency, which is calculated on the mean of the ingoing and outgoing humidity (Graph 1). In the same graph are shown the results of a number of earlier experiments in which the regulation of humidity was not good, though the experiments help to delimit a zone in which low humidity prevents the development of fleas of the F generation. The graph is far from complete, but gives a forecast of the type of quantitative information which may perhaps become available now that technical difficulties have been overcome.

GRAPH 1.



Showing production of F fleas per parental ♀ per day. Black circles ● indicate experiments in which bedding became mouldy and no F fleas emerged. Black dots • indicate successful experiments, and production of offspring per parental day. White circles ○ indicate conditions under which no offspring were produced.

Graph 1 also includes the result of certain experiments conducted at high humidity, in which the bedding became wet and mouldy. In these experiments no F fleas were produced. The conditions are perhaps quite unnatural, and due to working in an impervious glass jar. But it would be interesting to know whether something similar occurs in nature, in wet countries with a heavy soil; also whether rats' nests which have become mouldy produce fleas or not.

TABLE III.

The table relates to the production of fleas of the F generations, under certain conditions of temperature and humidity. It gives also the period from the start of the experiment to the emergence of the F fleas.

Reference.	Temperature, °C.	RELATIVE HUMIDITY.		Sat. def. (mean), mm. Hg.	Period, days.	♀ days excluding days 0 and 1.	OFFSPRING (F°).		EMERGENCE (DAYS).		80 per cent of fleas.
		Ingoing.	Outgoing.				Total.	Per ♀ day.	1st flea.	Last flea.	
P ..	24	85-91	91-100	2.3	6	117.8	181	1.53	39	52	40-48
Q ..	24	74-84	84-93	3.3	7	92.0	63	0.68	47	63	50-56
S ..	24	60-61	80-82	6.7	7	113.0	63	0.56	38	56	42-53
N ..	28	56-62	64-72	11.3	9	99.5	106	1.06	27	40	29-38
O ..	28	67-69	80-93	5.7	7	104.1	209	2.00	23	44	24-34
U ..	32	67-68	68-73	11.4	7	73.9	162	2.19	23	38	24-32
V ..	32	73-77	75-78	8.9	7	138.1	845	6.12	19	35	21-31

The last three columns of Table III give the number of days up to the emergence of the F fleas. The days are calculated from that on which the young P fleas first entered the experiment (day 0). This should be borne in mind in using the table, e.g., in experiment P the first fleas emerged on the 39th day, but assuming that the P fleas laid their first eggs on day 2, and that these eggs gave rise to the first emergences in the F generation, duration of the early stages is 37 days. It is clear from the table that the period spent from egg laying to adult depends partly on temperature, but that humidity is also an important factor, for there is a tendency for the F fleas to emerge sooner in the experiments in which the humidity was higher. These figures may be compared with those of Leeson (1932b); his technique was more precise than mine in that he used eggs under 24 hours old, incubating at 22°C. and 80 to 90 per cent relative humidity. He obtained his first emergences (always female) on day 36. This I regard as a more accurate figure than my own, but the difference between them is not great.

DISCUSSION.

The investigator's purpose was to carry out experiments in which conditions for rodent and flea are nearly natural, though capable of being measured and controlled. The work, therefore, falls into an intermediate position, and there are several sides from which it might be considered; on the one hand it relates to precise physiological research carried out *in vitro* and dealing with such subjects as the loss of water from parts of the insect; on the other hand it might help to explain observations made in the field on the prevalence of fleas and their relation to outbreaks of plague. Nor should it be forgotten that such experiments as these may perhaps lead to a more critical study of the flea count so widely used in the study of plague.

Relation to physiology.

We may first consider the relation between these experiments and the physiology of fleas, so far as it is at present known. One of the principal conclusions reached (Table I) is that the original adult fleas (P) survive about equally well at temperatures from 24°C. to 32°C., at least for the periods of the experiments. This appears to mean only that the insect is relatively long lived, for one must attribute the majority of deaths to the mice. It has also been established that humidity has an unimportant influence on survival for the period of the experiments. This is consistent with other work. For instance, Leeson (1932a, 1936) took large numbers of adult *X. cheopis* of known age; some were unfed, others had fed once and others had lived with a mouse for one week. These insects were exposed to controlled conditions of temperature and humidity without any further feeding, and it was found that duration of life was determined mainly by temperature and that the effect of humidity, though definite, was relatively slight. He established, moreover, that in fleas in several different stages of nutrition survival was by no means proportional to saturation deficiency, which appears to indicate that the loss of water by evaporation, even under the extreme conditions of some of the experiments, was a minor matter. In this respect one cannot reconcile Leeson's

work with that published by Bacot and Martin (1924). Their experiments were also carefully carried out, though the range of conditions which they covered was much less than Leeson's. Their conclusion that the duration of life was directly proportional to saturation deficiency, a conclusion which many of us have regarded as of great theoretical importance, appears to be no longer tenable. Whatever the effect of humidity, it may be supposed that under natural conditions any water which is evaporated from the flea's body into dry air would be made good the next time it fed. This has been fully demonstrated for *Cimex* (Mellanby, 1935).

The work of Wigglesworth (1935) helps to explain the adult flea's resistance to low humidity. When the insect is at rest the majority of the spiracles are shut and only two are in use; moreover, even they are only open intermittently. The general result of this regulation of the spiracles is to economize water, for the tracheoles are freely permeable to water, so that water must inevitably be lost by evaporation and diffusion, as long as the spiracles remain open. The importance of spiracular regulation in controlling loss of water was also clearly brought out by Mellanby (1934). In adult fleas exposed to 5 per cent CO_2 the spiracles may be seen to be permanently open and the rate of loss of water is doubled. The fact that adult fleas are so resistant to exposure to low humidities, even at high temperatures, shows the efficiency with which the loss of water from the respiratory system is controlled; it also shows that loss by evaporation through the general surface of the body is slight.

But resistance to dryness is not a result of respiratory control alone. A serious loss of water from the body might occur if the adult flea failed to extract water from the contents of the hind gut. The process of extracting water from the contents of the hind gut has also been described by Wigglesworth (1932). According to that author the flea possesses a distensible rectum, in the walls of which there are six rectal glands. If the flea has taken a small meal it may be seen that the blood is confined to the mid gut; fluid is rapidly absorbed from the mid gut and excreted by the Malpighian tubes; this fluid accumulates in the rectum and distends it. As digestion proceeds one must assume that the excretion and absorption of fluid is continuous. This conclusion that the rectal glands are responsible for absorbing water from the contents of the last part of the hind gut is supported by evidence derived from many other types of insect.

It is clear that the adult flea is indifferent or nearly so to humidity, even when low humidity and high temperature are combined, and recent work by insect physiologists has supplied a precise and reasonable explanation of this observation. But my experiments were sufficiently prolonged to give rise to offspring of the next generation (F): observations set out in the body of the paper have shown how differently this generation is affected by the climatic conditions provided in the apparatus, for it is clearly shown that humidity has a great effect on the production of fleas of the F generation, and that when it is below a certain figure no offspring are produced (Table III and Graph 1). As we already know that this cannot be due to the unfavourable effect of humidity on the adults, we must assume that the explanation lies in the physiology of the early stages, and it is necessary to review what is known about their relation to humidity. With regard to the egg, it is not known whether it is affected by differences in humidity, and it is possible that the egg changes in this respect as the embryo develops. As to the pupa, it is known that

even if it is extracted from the cocoon it is resistant to low humidity, much as the adult is (Mellanby, 1933). But the physiology of the larva is unlike that of other stages of the insect. Mellanby (1932) has shown that the highest temperature at which it can exist for one hour is $40.5^{\circ}\text{C}.$, and that this is not affected by humidity. But on a 24-hours' exposure the effect of humidity is very great; in air which is close to saturation the larva can survive $36^{\circ}\text{C}.$, but no higher temperature, but at lower humidities there is a regular fall in the thermal death point so that in dry air larvæ can only survive $22.5^{\circ}\text{C}.$ (Mellanby, 1932). Several observations serve to explain this. For one thing it is known that the extraction of fluid from the contents of the hind gut is much less efficient in larva than in adult (Wigglesworth, 1932). We may also suppose that the regulation of loss of water from the spiracles is less perfect in the larva. This seems to follow from the work of Mellanby (1934), who developed a method for directly weighing the water given off by batches of these insects during exposure to ordinary air, or to mixtures of gases. Working with adult *Xenopsylla* he found that the output of water in an atmosphere containing 5 per cent CO_2 was double that in air at the same humidity, and explained this as due to the spiracles being held permanently open, as actually observed by Wigglesworth (1935). But on treating larvæ in the same manner, no increase in loss of water was observed in 5 per cent CO_2 . In describing his results Mellanby made use of existing information, which appeared to indicate that the larva lacked any spiracular closing mechanism. But Sharif (1937*b*) has recently figured and described a closing mechanism, present in all the abdominal spiracles of the larva of *Nosopsyllus* (*Ceratophyllus*) and presumably also in *Xenopsylla*. Probably one can best reconcile this with Mellanby's work by supposing that the closing mechanism is less efficient in the larva than in the adult. It is also possible that the larval cuticle is permeable to water, and that some is lost through it. It seems then that the flea larva is killed by low humidities, at all temperatures, because it is not efficiently protected against excessive loss of water, either by the structure of the spiracles or of the hind gut.

The present experiments also indicate that even within the zone of conditions favourable to larval life the exact degree of humidity has a considerable influence on the rate of growth and the production of offspring; consider, for example, experiments *N* and *O*, both carried out at $28^{\circ}\text{C}.$:—

Experiment.	HUMIDITY, PER CENT.		Sat. def. (mean), mm. Hg.	Offspring (F) per ♀ per day.	1st emergence (F) day.	80 per cent emergence between days.
	Ingoing.	Outgoing.				
<i>N</i> ..	56-62	64-72	9.9	1.06	27	29-38
<i>O</i> ..	67-69	89-93	5.7	2.00	23	24-34

Similar figures were also obtained at $24^{\circ}\text{C}.$ and $32^{\circ}\text{C}.$ (Table III). At each temperature the production of F fleas was greater and more rapid at higher than lower humidities.

These differences in productivity and rate of development may be due to some effect of humidity on the larva itself, but there is another possibility which should not be forgotten. The organic matter which forms the food of the larva comes into equilibrium with the humidity of the atmosphere and will therefore contain more water at higher humidities. It is not improbable that it is only when the proportion of water exceeds a certain figure that the food can be easily taken in and digested: it may be for this reason that humidity not only affects the production of offspring, but also the duration of early stages. Sikes (1931) investigated a number of different substances on which flea larvæ might feed, and showed that they differed from one another in their hygroscopic powers. She suggested that one essential factor in larval growth was a particular concentration of water in the food, and that therefore larvæ would thrive at different humidities on different materials. This interesting point requires to be re-investigated now that the work of Sharif (1937*a*) has supplied fuller information about the nutrition of the larvæ.

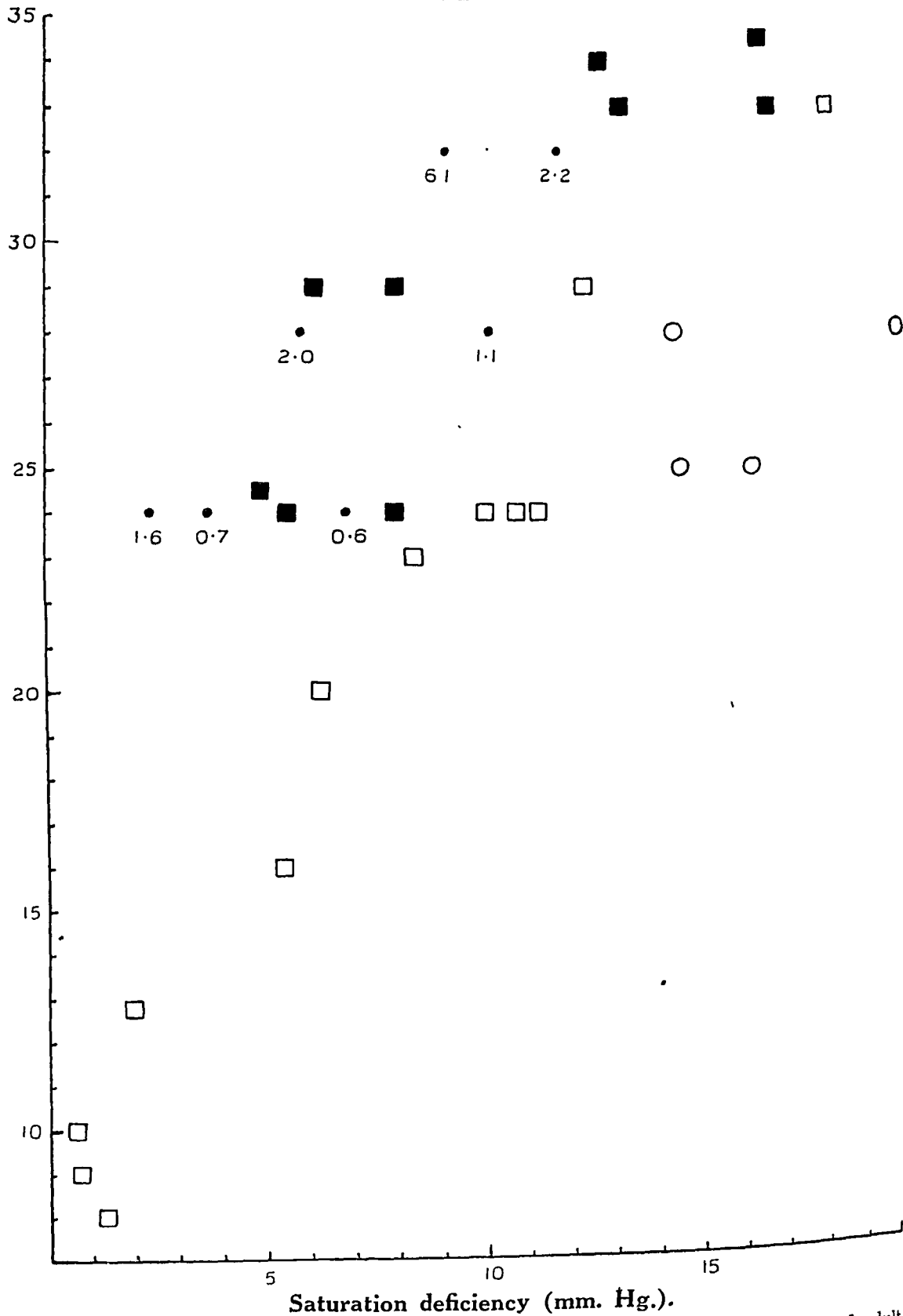
The physiologists have tended to focus our attention on the larva of the flea, and their work shows that the conditions which suit it are narrower than those which the pupa or adult can tolerate. It is legitimate, therefore, to make use of other work on the effect of humidity on the larva to supplement what has already been set out in Figure 4; the largest collection of information is given by Bacot (1914—Tables 19 and 20). Bacot took larvæ, partly or fully grown, from a culture jar and exposed them to conditions of temperature and humidity, his methods of measurement and control being, it must be confessed, not so precise as those which are used nowadays. He delimited the conditions of temperature and humidity favourable and unfavourable to the production of adults from these larvæ. Bacot's data and those given in this paper are brought together in Graph 2 and it will be seen that they are consistent with one another. It seems that the line dividing the favourable and unfavourable zones is rather sharp; it is clear also that this line runs obliquely across the graph. This is as would be expected, for at different temperatures the duration of larval life, and therefore of exposure to risk of drying up, is for different periods. One would, therefore, not expect that some particular value of saturation deficiency would delimit the tolerable conditions over a range of temperature; more probably the product of length of life (days) by highest tolerable saturation deficiency (mm. Hg.) would be a constant (see Mellanby, 1933). Whether this is so cannot yet be decided in the absence of data on the length of larval life at different temperatures.

It is hardly possible to close this part of the discussion without pointing out how closely the observations made on the biology of the whole animal and the work of physiologists who have studied its parts and system are consistent with one another.

Relation to field work.

It is now a good many years since Brooks (1917) examined the statistics of plague from many places in Northern India and a few elsewhere and formulated certain general rules. He found that plague does not maintain itself in epidemic form when the temperature (in the screen) rises above 80°F. (26·7°C.) accompanied by a saturation deficiency of over 0·3 inch (say 8 mm.); but even if the temperature is higher, provided the saturation deficiency is less, plague may commence

GRAPH 2.



Showing conditions favourable (black) and unfavourable (white) to production of adult *Xenopsylla cheopis* from larvæ. The circles are taken from Graph 1 (above), the squares from Bacot (*loc. cit.*).

and increase in intensity. These climatic limitations have found a general acceptance, and it is held by most workers, for instance, Hirst (*loc. cit.*), that they are due, at least in the main, to the effect of climate upon the fleas which transmit the infection. As a broad rule the figures arrived at by Brooks are probably near the truth, but as a result of recent advances in the physiology of the insect, we now suppose that the limit of dryness will not be some particular value of saturation deficiency; on the contrary, at higher temperatures the larvæ will be found to tolerate dryer air because they develop more quickly, and are therefore exposed for a shorter time (*see above*). A considerable body of fact has been produced in support of this view (Graph 2). It seems, moreover, that fleas not only exist but are capable of rapid multiplication when the air is much dryer than a saturation deficiency of 8 mm.; for instance, at 32°C. and a saturation deficiency of 11.4 mm. I have found a production of 2.19 adult offspring per parental female per day. Bacot (*loc. cit.*) also obtained a considerable proportion of adults from larvæ at 33°C. and 34°C. and in air which was still dryer, though his regulation of humidity was not precise. One would hardly expect the insects to breed successfully at temperatures much higher than these, for it is shown by Mellanby (1932) that the highest temperature which the adult can survive for a period of 24 hours is just below 38°C.

It must be remembered that though fleas multiply so rapidly at temperatures exceeding 30°C. it does not follow that transmission of plague would be most common at those temperatures. It seems probable that the climatic optimum for the survival of infected fleas is cooler.

It is clear that work tending to produce a quantitative biology of the insect under conditions which are precisely defined, will have much greater practical value when we know more of the climatic conditions which exist in the spots where the insect itself lives. The epidemiologist, engaged in forecasting the course of plague epidemics, needs a considerable body of fact about the temperature and humidity in the holes, haunts and nests of the rats. The objection will at once be put forward that the conditions of a rat's nest in a wall are quite unlike those in a nest in the roof, and again quite unlike those in a hole in the ground; also that even in the ground conditions will be quite different in the open, under a building, or in the north or south side of a courtyard. The writer is fully aware of these difficulties, but urges that it is necessary to make these investigations and to continue them for a period of several years. The technical difficulties in measuring the temperature and humidity are not any longer very great. When a considerable body of fact has been collected about climatic conditions in rat holes, etc., it may become possible to apply corrections to standard meteorological data, and make better use of them in relation to the epidemiology of plague.

It seems that very few attempts have been made to work along these lines. One investigation was limited to a short period in the summer in Palestine (Buxton, 1932). But enough was done to reveal the complexity of the subject and define a few general conclusions. It was found that, doubtless owing to the effect of radiant solar heat, the temperature in buildings and in the surface of the soil was frequently much higher than that in a Stevenson's screen. In holes and such places the temperature was extremely stable. Conditions of humidity varied greatly from place to place. In cellars, stables, and rat-holes, in which evaporation

was actively going on, even though no rain had fallen for many months, the absolute humidity was much higher than it was outside. In most cases the saturation deficiency in these small environments was very much below that recorded in the screen, and the conditions for flea breeding presumably more favourable.

It appears that experiments such as are described here might lead investigators of plague to consider the 'flea count' from a new point of view. The figures set out in Table II show that there is a large variation in the proportion of fleas found on the mouse's body at the end of an experiment; moreover, the variation appears to be unrelated to temperature or humidity. The gross percentage of fleas on the mouse is 12·8, the range in groups of experiments (not in individual experiments) running from 4 to 28 per cent. These figures are curiously different from those published by Leeson (1936). For his work he required *X. cheopis* which had been kept with a mouse for a week, and he recorded the number found on the mouse and in the bedding; in 19 experiments taken together, of 1,200 fleas, 42 per cent (505) were on the mouse, but in individual experiments the proportion ranged from 17 to 89 per cent. There is another discrepancy between these figures and Leeson's in the proportion of the sexes found on the mouse. In two groups of experiments (Table II) the females were significantly more numerous than the males ($P < 0\cdot01$). Leeson, adding the results of his experiments together, observed out of 717 males 46 per cent, and out of 483 females 38 per cent, on the mouse; the difference is significant ($P = 0\cdot02$), though the reverse of that found by myself. Leeson has also recorded that many of his individual experiments differed widely from one another in this respect. Webster and Chitre (1930) report on the position of live fleas in cages containing rats recently dead of plague. The proportion of *cheopis* on the rat is consistently greater in the 'off season' than in the 'plague season'. As is recorded in the Postscript, the proportion of fleas found on a baby mouse is much higher than on an adult. The rodent's age is therefore an important variable.

It seems that in this respect the results of experimental work are inconsistent with one another. Surely some investigator of plague should find it worth while to give further study to this point. With no more equipment than a number of battery jars, each containing a rat and a population of fleas, it might be determined whether the number on the rat varied with season and whether it would be affected by the type of bedding, size of the container, age of rat, light, temperature and other factors which could readily be varied. One might in this way discover more precisely what it is that the flea count records.

The value of the flea count is very well established and generally admitted, and I am very far from wishing to bring it into disrepute. On the contrary, I suggest that the matter is important enough to demand study from a new angle, the experimental, so that we may acquire knowledge of the limitations of this widely used method. So far as present knowledge goes the flea count cannot be regarded as a good measure of natural populations of fleas. For one thing it is based on the assumption that the unknown proportion of fleas which are on the rat's body is approximately constant; but it is at least possible that this proportion alters with season, depending on the flea's hunger or activity; it may also be different for fleas of different age, sex or state of nutrition. Moreover, one

should not overlook the fact that changes in the rat population would tend to dilute or concentrate the population of fleas, if their numbers remained unaltered.

SUMMARY.

1. The paper describes an apparatus in which a rodent and a population of fleas may be maintained under conditions which are almost natural and at the same time capable of control and measurement.

2. The survival of the P (parental) fleas is erratic; this is mainly due to the mouse eating the insects, as the Postscript shows. It appears that survival, up to 7 days, is not affected by temperature or humidity. This is consistent with what is known of the physiology of the adult flea. It is probably true to say that its loss of water, even at high temperature and low humidity, is insignificant, and that the loss can be made good at the insect's next meal.

3. The proportion of P fleas on the mouse and in the bedding at the end of an experiment varied greatly. The differences did not appear to be related to climatic conditions. There was a tendency for a higher proportion of ♀ ♀ fleas to be on the mouse. The flea count, a technique used so widely, should be submitted to experimental study.

4. Fleas of the F (first filial) generation were bred out, the material being kept under the same climatic conditions as those to which the parental fleas had been exposed. No emergence of F fleas occurs when the humidity is below a certain saturation deficiency, which is not the same at all temperatures. Even at one temperature and within tolerable limits of humidity, the production of F fleas is closely dependent on humidity, the production being less as the fatal limit is approached. The period occupied by the early stages is also influenced by humidity, probably because the growth of the larva is retarded when the water content of the food is low.

5. These observations on the production of F fleas are consistent with what is known of the physiology of the larval stage. The larva is not capable of much resistance to desiccation, losing water with its excrement, and probably also in respiration (in spite of possessing a spiracular closing device).

Postscript.—Though most of the major variables were successfully controlled, the number of P fleas which survived varied greatly and erratically (Table I): this was thought to be due to the mouse catching and eating many fleas.

After the work was finished, at the suggestion of the writer's colleague, Dr. M. Watson, we substituted a single baby mouse for the adult; the mice were six to nine days old, just able to crawl, and one fresh baby mouse was used each day. The experiments were carried out in jars with a diameter of 5 inches. The substitution of baby mouse for adult made a very great difference in the proportion of fleas (*X. cheopis*) surviving. The results obtained are shown in Table IV. The figures seem to indicate that, as previously suspected, adult

mice eat a considerable number of fleas. It will also be noticed that a high proportion of the surviving fleas were found on the body of the baby mouse at 31°C. These observations will enable us to eliminate another important variable in future experiments.

TABLE IV.

Showing results obtained by keeping equal numbers of Xenopsylla cheopis with a baby and an adult mouse.

Temperature, °C.	Number of experiments.	Fleas per experiment.	SURVIVING FLEAS, 7TH DAY.		PROPORTION OF FLEAS ON BODY OF MOUSE, 7TH DAY.	
			Baby.	Adult.	Baby, per cent.	Adult, per cent.
31	S	50	48-50	2-26 mean 14.1	55	21
25	S	50	47-50	17-50 mean 25.7	30	39

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ON THE NUMBER OF EGGS OF THE COMMON HOUSE-FREQUENTING FLIES OF CALCUTTA.

BY

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[Received for publication, May 23, 1938.]

ALTHOUGH we have some data as to the number of eggs that *M. domestica* can lay at a time, we have none in regard to any of the common Indian domestic flies, especially *M. vicina*, the commonest of the house-frequenting flies in this country. It is not the intention here to enter into any controversy whether *M. vicina* and *M. domestica* are the same species or different; one may note, however, that Patton (1931) has brought forward convincing evidence to show that they are not the same.

The number of eggs that a fly can lay in a single batch can be determined either by counting the eggs when they are laid or by their number in the ovaries of gravid females.

The former method is difficult to follow for the reason that there is a tendency on the part of the fly to deposit eggs in very small nooks, cracks, and holes, where it is not possible to count them accurately. The latter method, though it seems theoretically sound, has not yet been tested in practice. It was the object of this inquiry to test it in trying to find out the number of eggs that a fly usually lays in a batch.

Three species of domestic flies of Bengal, *M. vicina*, *M. nebulosa*, and *Chrysomya megacephala*, were experimented with.

Table I represents the number of mature eggs which were detected in the ovaries of gravid females after dissection. The adults were all caught in bazaars.

TABLE I.

NUMBER OF EGGS FOUND IN THE OVARIES.				
		Average.	Maximum.	Minimum.
<i>M. vicina</i>	..	97	134	60
<i>M. nebulo</i>	..	75	81	65
<i>C. megacephala</i>	..	182	211	136

Hence on an average a single *M. domestica*, *M. nebulo*, and *C. megacephala* can deposit 97, 75, and 182 eggs, respectively.

The dissections in the above series were performed in March and April, at a time when it is supposed that the rate of their multiplication takes place at a very rapid pace. During the winter season, on the other hand, they show a tendency to produce a smaller number of eggs, and the proportion of gravid females among the wild catches at that time is considerably less than during summer. Thus, the number of eggs that was found in the ovaries after dissection in December was 72, 64, and 166 in *vicina*, *nebulo*, and *megacephala*, respectively.

No accurate knowledge was previously available as to whether or not the ovaries completely discharge their contents when a batch of eggs is laid. This was ascertained by dissection of the ovaries of *M. vicina* after oviposition, and as no egg was detected in the ovaries in a single instance, it therefore followed that when once egg-laying starts, there is a tendency on the part of the fly to empty the contents of the ovaries as quickly as possible. In contrast to such a finding one may cite the experience of Dunn (1923) when he reported from the Panama Canal Zone that *M. domestica* was capable of producing large batches of eggs almost daily. He experimented with what appeared to be an extremely prolific race of flies of that species. He left a pair of them in each lamp globe with fresh horse dung every day to deposit their eggs in, and milk was given to them for their food. Whether such introduction of horse manure might have a stimulating influence on their egg production, which could account for an almost continual production of eggs every day or at an interval of a day, was also determined by me.

For this purpose an experiment was planned out on the lines carried out by Dunn, with the only difference that *gur* was substituted for milk and extra water was provided. The horse manure was changed daily for four days in succession, and at the end of every 24 hours the samples were carefully examined for the presence of eggs or larvæ. It was noticed that under such conditions once the flies had deposited a batch of eggs, further depositions within a period of three days did not take place, and in this respect there was a sharp difference in results from those recorded by Dunn.

The prolificity of *M. vicina* and *M. domestica* can now be compared :—

TABLE II.

<i>M. vicina.</i>	<i>M. domestica.</i>
97 in March and April	120 or perhaps more (Howard, 1912).
72 in December	120 to 125 (Forbes of Illinois, cited by Howard, <i>loc. cit.</i>).
	120 (Hewett, 1914).
	120 to 140 (Newstead, 1908).
	22 to 159 (Dunn, <i>loc. cit.</i>).

Thus, in respect of egg-laying capacity, *M. vicina* departs widely from its European prototype.

SUMMARY.

The significant facts that have emerged out of this investigation are :—

1. Counting of eggs in ovaries of gravid females affords an easy means of ascertaining the number of eggs that the fly is capable of depositing in a batch.
2. The three common species of house-frequenting flies in Bengal, e.g., *M. vicina*, *M. nebulosa*, and *C. megacephala*, lay on an average 97, 75, and 182 eggs, respectively, in one laying.
3. Though morphologically *M. domestica* and *M. vicina* resemble each other, they differ in regard to the number of eggs they usually deposit in a batch.
4. When one batch of eggs is laid, some time must elapse before another batch becomes mature.

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A NOTE ON THE OVERWINTERING OF THE HOUSE FLY, *MUSCA DOMESTICA*.

BY

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[Received for publication, June 10, 1938.]

IN Muktesar (U. P.) during the winter of 1937-38 the house fly, *Musca domestica*,* was commonly seen both in houses and animal sheds. This led to the investigation of the nature of overwintering as there has been some dispute over this phenomenon in the case of house flies (Patton, 1931).

The question arose whether the house flies noted in the winter season from November 1937 to March 1938 in places such as Muktesar situated at an altitude of 7,500 feet above sea-level were really hibernating flies which came out of their winter sleep on comparatively warm days or whether they indicated that reproduction of flies continues even in winter. In the experience of Kobayashi (1934, 1935), the house fly (*M. domestica*) does not hibernate in the Far East.

House flies were frequently caught and examined during the months of December, January, February, and March. In these collections both sexes were represented (the ratio being two males to three females) and the flies were quite active. There was no excessive accumulation of fat bodies in the abdomen of these flies. The ovaries in the females were noted to be fully developed in some but in the great majority they were very poorly developed. The flies usually frequented such places as animal sheds and sculleries where food was plentiful. They invariably disappeared as soon as the warm spell was over and they were found to be very adversely affected by a drop in the temperature of the room. All these observations suggested that the house flies, *M. domestica*, in Indian hills do not hibernate. If these were merely hibernating flies coming out of their winter retreats owing to warm spells, they would all have been females as has been noted in several other truly hibernating insects such as *Pollenia rudis* (Calliphorinæ), *Culex pipiens*, and *Anopheles maculipennis* (Patton). The ovarian development would have been suspended and the flies would be provided with a reserve store of fat. Hibernating flies would never be killed by the freezing cold of the winter. These would be negatively heliotropic and would not come out in the light as was

* Type species as determined by the key of Patton and Senior White.

noted in case of the house flies occurring in Muktesar. The view that the house fly overwinters by constant breeding in warm places appears to be established.

This view has been further supported by observation of actual oviposition by *M. domestica* on 24th January and again in the latter half of February (1938). On both these occasions the eggs were laid on the 'glass tubes in which the house flies were kept and egg laying started immediately after capture. The number of eggs laid was small. On the first occasion only one egg was noticed and from this hatched out almost immediately the tiny maggot, reminding one of the viviparous mode of reproduction in some blood-sucking muscids. In the second instance about 7 or 8 eggs were laid but these failed to hatch presumably on account of the low temperature.

The small number of eggs and the very appreciable reduction in the length of time required for the egg to hatch are probably provisions to ensure better chances for the species to survive the winter when conditions for the propagation of the flies are very unfavourable.

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CHEMOTHERAPEUTIC STUDY OF STREPTOCOCCAL INFECTIONS.

BY

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[Received for publication, May 23, 1938.]

RECENT researches on the chemotherapy of streptococcal infections have shown that p-amino-benzene-sulphonamide renders the blood antiseptic to different types of hæmolytic streptococci, but the drug is to be usually administered in large doses which is often difficult and sometimes leads to toxic effect. Buttle *et al.* (1936) have again demonstrated that the bactericidal property of the above compound is not exclusively due to the presence of the sulphonamide grouping, SO_2NH_2 , and is even found in certain diphenyl sulphones (Buttle *et al.*, 1937). Consequently, it was considered to be of interest to make a further study on other substances more or less closely related to the p-amino-benzene-sulphonamide.

Accordingly, with the idea that linking the amide group with other chain or ring known to possess or exert chemotherapeutic activity might lead to the isolation of a better product, 4-amino-benzene sulphon- δ -diethyl-amino-butyl-amide, 4-amino-benzene sulphon-8'-quinolyl-amide and 4-amino-benzene sulphon-6'-methoxy-8'-quinolyl-amide have been recently prepared and described by one of us (Choudhury *et al.*, 1937). The bacteriostatic and the bactericidal actions of the above compounds are now embodied in the present paper.

It has been found that 4-amino-benzene sulphon-8'-quinolyl-amide possesses a bacteriostatic action on hæmolytic streptococci *in vitro* in a concentration of 1 in 4,000 and a test on mice indicates that the compound is extremely toxic. The other two products have no such action even in a concentration of about 1 in 1,300. 4-amino-benzene sulphon- δ -diethyl-amino-butyl-amide is also equally toxic to mice.

EXPERIMENTAL.

Groups of white mice (weighing from 10 g. to 12 g.), three in each case, were infected with broth cultures of streptococcus hæmolytic and these were injected

intramuscularly with different doses of 4-amino-benzene sulphon- δ -diethyl-amino-butyl-amide and 4-amino-benzene sulphon-8'-quinolyl-amide in the form of their dihydrochloride salt solutions. Six animals were only infected but not treated with the compounds and they served as controls in each case. All of them were kept under observation for 96 hours and the results are shown in Table I:—

TABLE I.

Drug:—Dihydrochloride of 4-amino-benzene sulphon- δ -diethyl-amino-butyl-amide, or 4-amino-benzene sulphon-8'-quinolyl-amide.

Number of mice:—24, 6 being for control in each case.

Infecting dose:—0.25 c.c. of 18 hours' broth culture of strepto-hæmolytic.

Number of mice.	Infecting dose in c.c.	Dose of the drug in mg.	Result.
3	0.25	7.5	All dead.
3	0.25	6.0	
3	0.25	4.5	
3	0.25	3.5	
3	0.25	2.5	
3	0.25	1.5	
6 (control)	0.25	..	

It was next considered to be of importance to find out whether the drugs themselves are toxic for the mice. Accordingly, groups of six mice, three in each case, weighing 10 g. to 12 g. were taken and injected intramuscularly over the flanks in different doses with each of the above two compounds rendered soluble by means of hydrochloric acid. The results in each case are as recorded in Table II:—

TABLE II.

Number of mice.	Dose of the drug in mg.	Result.
3	7.5	All dead.
3	6.5	
3	4.5	
3	3.5	
3	2.5	
3	1.5	

From Table II it is evident that both the drugs in the form of their hydrochloride salts are fatal for the mice even in dosage of 1.5 mg.

In order to find out the bacteriostatic action on cultures of strepto-hæmolytic, all the drugs mentioned, being almost insoluble in water, were first of all passed through a 200 sieve and then were made up to 1 per cent suspensions in water containing 0.2 per cent of tragacanth. Ordinary nutrient broth 10 c.c. were inoculated with the organisms and, after half an hour, different amounts of all the three drugs in suspension were added to the test-tubes containing the cultures. To get an uniform result three sets of tubes were used for each amount of the different drugs, and they were all incubated at 37°C. for 18 hours. The results obtained are given in Table III:—

TABLE III.

Culture :—Strepto-hæmolytic in 10 c.c. nutrient broth.

Drug in suspension.	AMOUNT IN MG.							Control: 1 c.c. of 0.2 per cent tragacanth.
	0.25	0.5	1.0	2.5	3.5	5.0	7.5	
-Butyl-amide ..	Growth	Growth	Growth	Growth	Growth	Growth	Growth	} Growth.
-6'-methoxy-8'-quinolyl-amide.	"	"	"	"	"	"	"	
-8'-quinolyl-amide	"	"	"	No growth	No growth	No growth	No growth	

Thus, it is evident that only 4-amino-benzene sulphon-8'-quinolyl-amide inhibits growth of strepto-hæmolytic when 2.5 mg. in tragacanth suspension were added to the test-tube containing 10 c.c. of the culture. The other products had no such action even in the concentration of 1 in 1,333.

DISCUSSION.

The results indicate that the compounds under investigation are too toxic for the mice. Of course, the drugs in question were used in the form of their hydrochloride salts in which form p-amino-benzene-sulphonamide is also known (*cf.* Buttle *et al.*, 1936) to produce untoward symptoms. But as 4-amino-benzene sulphon-8'-quinolyl-amide is found to possess bacteriostatic action only in a concentration of 1 in 4,000 [p-amino-benzene-sulphonamide being active in a concentration of 1 in 18,000 (De and Basu, 1937)], it would not be of any worth to study bactericidal effect of the drug itself on animals via the alimentary tract. The other two have no action even *in vitro*. It may be pointed out here that whereas the chemotherapeutic activity is enhanced by the replacement of an

amino group by an amino-alkyl-amino chain in certain antimalarial drugs (*cf.* Schulemann *et al.*, 1926, 1932), the linking of such a chain to the amide group of sulphonamide has not, however, resulted in any increase in the bacteriostatic activity of this latter drug. But since 'Prontosil' has been found to be effective (Virgil *et al.*, 1937; Hill and Goodwin, 1937; Read and Pino, 1938) even in the treatment of quartan malaria, it would be interesting to study the effect of these compounds on malarial parasites. Work in this direction is already in progress.

SUMMARY.

The replacement of the amide hydrogen of p-amino-benzene-sulphonamide by 8-quinolyl, 6-methoxy-8-quinolyl, or δ -diethyl-amino-butyl group, each of which is known to enhance the physiological activity of a chemotherapeutic compound, does not help in the increment of the bacteriostatic property of sulphonamide; on the contrary, it increases the toxicity of the compound.

ACKNOWLEDGMENT.

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HÆMATOLOGICAL STUDIES IN INDIANS.

Part IX.

THE ANALYSIS OF THE HÆMATOLOGICAL FINDINGS IN 57 CASES OF ANÆMIA IN PREGNANT TEA-GARDEN COOLIE WOMEN, WITH SPECIAL REFERENCE TO THE RESULTS OF TREATMENT.

BY

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THIS paper is a report of further investigation into the ætiology of the anæmia prevalent amongst tea-estate labourers.

In an earlier investigation, we (Napier and Billimoria, 1937) made a fairly complete examination of the blood picture in 52 cases of pregnancy anæmia, but in that series in the majority of cases only one examination was made. In the present investigation, we have followed up the patients for as long as possible to ascertain the reaction to various forms of treatment.

Technique.—The technique adopted was the same as that in our previous investigations. The actual examinations were carried out by the junior writer (D. N. M.) who had been working in the senior writer's laboratory for about a year at the time of the commencement of this phase of the inquiry and had standardized his methods and instruments.

Blood examinations were carried out as frequently as possible, but at least once a week during the important periods of investigation. The reticulocyte count was done in most cases on alternate days, but in some of the graphs the peak only was recorded. In some instances it is feared that the peak was missed.

Controls.—It was considered advisable to examine the blood of a certain number of 'normal' coolies, as we had no normal data for the coolies in this district. For this purpose, 25 male and 25 female working coolies, between the ages of 20 and 40, apparently in good health, were selected and their blood examined. The blood findings are summarized in Table I, and certain other data are included in the appropriate tables below:—

TABLE I.

		Range.	Mean.	S.D.
Hæmoglobin in g. per 100 c.c.	Males	7.01 to 15.54	12.60	± 1.83
	Females	6.88 to 13.34	10.40	± 1.73
Red blood cells in millions per c.mm.	Males	3.88 to 6.05	5.057	0.563
	Females	2.98 to 5.53	4.454	0.705
White blood cells in thousands per c.mm.	Males	5.05 to 9.95	7.111	1.759
	Females	3.10 to 9.90	6.456	1.755
Cell volume, per cent	Males	28.34 to 51.23	42.18	4.81
	Females	17.99 to 47.60	37.07	7.34
Mean corpuscular volume (MCV)	Males	60.1 to 105.5	84.93	10.78
	Females	55.9 to 108.0	82.49	12.68
Mean corpuscular hæmoglobin (MCH)	Males	14.8 to 31.8	25.14	3.70
	Females	18.6 to 31.4	23.42	3.10
Mean corpuscular hæmoglobin concentration (MCHC).	Males	26.9 to 39.3	29.72	2.94
	Females	21.7 to 38.3	28.67	3.37

The figures for hæmoglobin, the red cell count, and the white cell count do not differ materially from those of the other coolie populations (Napier and Das Gupta, 1936; Napier and Billimoria, *loc. cit.*); the cell volume, however, is distinctly higher in the present series. This difference is better shown in the corpuscular values, the mean corpuscular volume (hereafter referred to as MCV) and the mean corpuscular hæmoglobin concentration (hereafter referred to as MCHC). In the present series, the former is markedly higher than in the two previous series, and the latter correspondingly lower, as the mean corpuscular hæmoglobin (hereafter referred to as MCH) is about the same in the three series.

The correct estimation of cell volume is dependent on two things, the anti-coagulant and the rate of centrifugalization. We have used 0.2 per cent potassium oxalate in all our estimations, and have multiplied our cell-volume findings by the factor 1.09 to compensate for shrinkage. This procedure has been questioned by Sokhey *et al.* (1937) who have found that their sample of potassium oxalate produces a lower degree of shrinkage. In one of the previous series, Napier and Das Gupta (1936), we used an efficient centrifuge, but in the other, Napier and Billimoria (*loc. cit.*), the centrifuge was defective and for this reason we made an allowance in our readings. In the present series the centrifuge was a good one but the electric supply was sometimes weak. If a centrifuge is revolving slowly, packing will never be complete, and this was certainly the case in some of our estimations. We believe, from evidence into which we need not go into now, that in this series the cell-volume estimations as a whole were about 5 per cent higher than they would have been if packing had been complete and, therefore, the MCVs will be 5 per cent too high and the MCHCs correspondingly 5 per cent too low, in both the anæmic cases and the controls; we have not adjusted our readings to counteract this error.

For this reason we are not laying as much emphasis on the volume of the cells as on their hæmoglobin content.

Compared with those of normal city-dwelling Indians—males, i.e., 14.77 g. (Napier and Das Gupta, 1935a), 15.70 g. (Napier and Das Gupta, 1936), and 15.37 g. (Sokhey *et al.*, 1937), and females, i.e., 14.55 g. (Sokhey *et al.*, 1938) and 12.52 g. (Napier *et al.*, unpublished)—the figures for hæmoglobin are very definitely (and statistically very significantly) lower. On the other hand, the number of red cells is very nearly the same as the above workers found in city-dwelling Indians, i.e., males 5.36, 5.53, and 5.11 millions, and females 4.47 and 4.671 millions. Thus, the mean corpuscular hæmoglobin is very much below normal, or, in other words, the hæmoglobin deficiency in the whole blood is dependent not on the number of cells but on the small amount of hæmoglobin in each individual cell.

This low mean corpuscular hæmoglobin has been a constant finding in all our investigations amongst tea-garden coolies.

From the data given by Napier and Das Gupta (1935a and b) the MCH for males can be calculated to be 22.1 $\gamma\gamma$ and for females 22.0 $\gamma\gamma$; in our latter series (Napier and Das Gupta, 1936) the figures were 23.91 $\gamma\gamma$ and 23.35 $\gamma\gamma$, respectively, and Napier and Billimoria (*loc. cit.*) found a mean of 24.51 $\gamma\gamma$ for non-pregnant and 23.80 $\gamma\gamma$ for pregnant female coolies. These are contrasted with 27.56 $\gamma\gamma$ (calculated) and 28.53 $\gamma\gamma$ for male city-dwelling Indians (Napier and Das Gupta, 1935, 1936), 30.08 $\gamma\gamma$ (Sokhey *et al.*, 1937) and 29 $\gamma\gamma$ which is the figure usually quoted for Europeans and Americans.

It is not quite certain how far this deficiency in the hæmoglobin of the individual cell is associated with a deficiency in size. Our earlier investigations suggested that this latter deficiency was as great but findings have not been quite consistent (*vide supra*). In the present series the population, though a tea-garden coolie force, is a different one from that of the earlier investigations and it is conceivable that the size of the cell may be actually, as well as apparently, larger. However, it is quite clear that in size the cell in this population is distinctly smaller than the normal.

What is the significance of this under-hæmoglobinized small cell is not certain. It is the usual blood picture of chronic hæmorrhage and iron deficiency, but we have attempted to raise the hæmoglobin in so-called normal coolies by giving large doses of iron and have failed (though the experiment would be worth repeating on a larger scale); on the other hand, Sankaran and Rajagopal (1938) successfully raised the hæmoglobin level in their Madras 'normals' by giving comparatively small doses of iron. Does the continued drain on the blood caused by hookworm infection exhaust the body reserves of some other substance necessary for normal hæmopoiesis in a coolie living on a minimal diet?

Discussion on normal standards.—The next problem is what is to be our attitude towards the hæmatological data obtained from the so-called normal coolie. Are we to judge the hæmoglobin of the individual female on the 14.0-gramme basis or on the 10.4-gramme basis? If on the former, then the majority of our so-called normal coolies would have to be considered anæmic. Or, if on the latter, then coolies with hæmoglobin above 7 grammes must be considered to be within the normal range; this may seem absurd but in actual practice, though no doubt their efficiency is low, they show few symptoms of anæmia and seldom apply for treatment until their hæmoglobin percentage is below this figure.

The same question arises in connection with the corpuscular values. Should the MCH of 28.53 ± 2.31 or 23.42 ± 3.10 be considered the normal? If the former, then again the majority of the so-called normals would have to be classed as hypochromic and, if the latter, $17.22 \gamma\gamma$ would be considered the upper limit for hypochromia, which would be absurdly low.

The patients.—The patients were tea-garden coolies employed in one or other of the gardens that are served by the Central Hospital, Dewan, near Silchar, under the medical charge of Dr. G. Fraser. They were sent in from outlying garden hospitals to the Central Hospital for special treatment and for investigation. Only the worst cases were selected for special treatment from distant gardens, but from the gardens close at hand all coolies that required hospital treatment were admitted to this hospital. For this reason, in this series, there are fewer patients with slight degrees of anæmia than in our previous series in which all anæmic pregnant women were included; in fact with one exception their initial hæmoglobin level was below 7 grammes per 100 c.c. of blood.

The attitude of the pregnant coolie woman is very fatalistic: she assumes, not entirely without reason, that any degree of ill-health which she is suffering is due to pregnancy and that all will be well when she is delivered. She, therefore, seldom applies for medical treatment unless some complication supervenes, and she will often continue to work with her hæmoglobin level at 3 grammes and even lower. The majority of these patients came under observation during the routine inspection of pregnant women, the remainder because they were suffering from some complications.

When asked she will admit to weakness but seldom to breathlessness, and beyond her anæmic appearance oedema of the feet is often her only obvious sign, though on auscultation a hæmic murmur may be heard. In a few cases the red, smooth tongue of the pellagrin was noticed, but there was seldom any soreness of the mouth; koilonychia was not noted. There was often a slight cloud of albumin in the urine but no casts.

A number of the patients were suffering from some complications, diarrhoea, bronchial catarrh or just 'fever' being the common complaints. A moderate degree of fever was very common and this often lasted for a week or so into the puerperium.

A history was often given of anæmia with previous pregnancies, but the patient usually said that it had not been so marked. Occasionally, however, there was a history of severe anæmia at each pregnancy.

Clinical classification.—The possibility of making a clinical classification was considered but soon abandoned. A recent writer (Mitra, 1937) has classified his cases according to present complications and histories of certain diseases in the recent past, but the procedure did not seem to us to be very successful.

In our cases, the histories regarding previous illness were unsatisfactory: we have discussed this point later in the paper.

Hæmatological classification.—The hæmatological picture presented by these patients is not a constant one, and, therefore, the first step should be an attempt to divide them into hæmatologically homogeneous groups.

The question that arises is, on what particular feature of the blood picture should the classification be based? In view of our uncertainty as to the absolute accuracy of our cell-volume estimations, we felt that it would be best to place more emphasis on the mean corpuscular hæmoglobin (MCH) than on the mean corpuscular volume (MCV).

Our plan in the last series (Napier and Billimoria, *loc. cit.*) was not to adopt any pre-conceived method of classification but rather to examine the blood pictures and to see if they appeared to arrange themselves into any distinct groups, and in this series we have adopted the same plan.

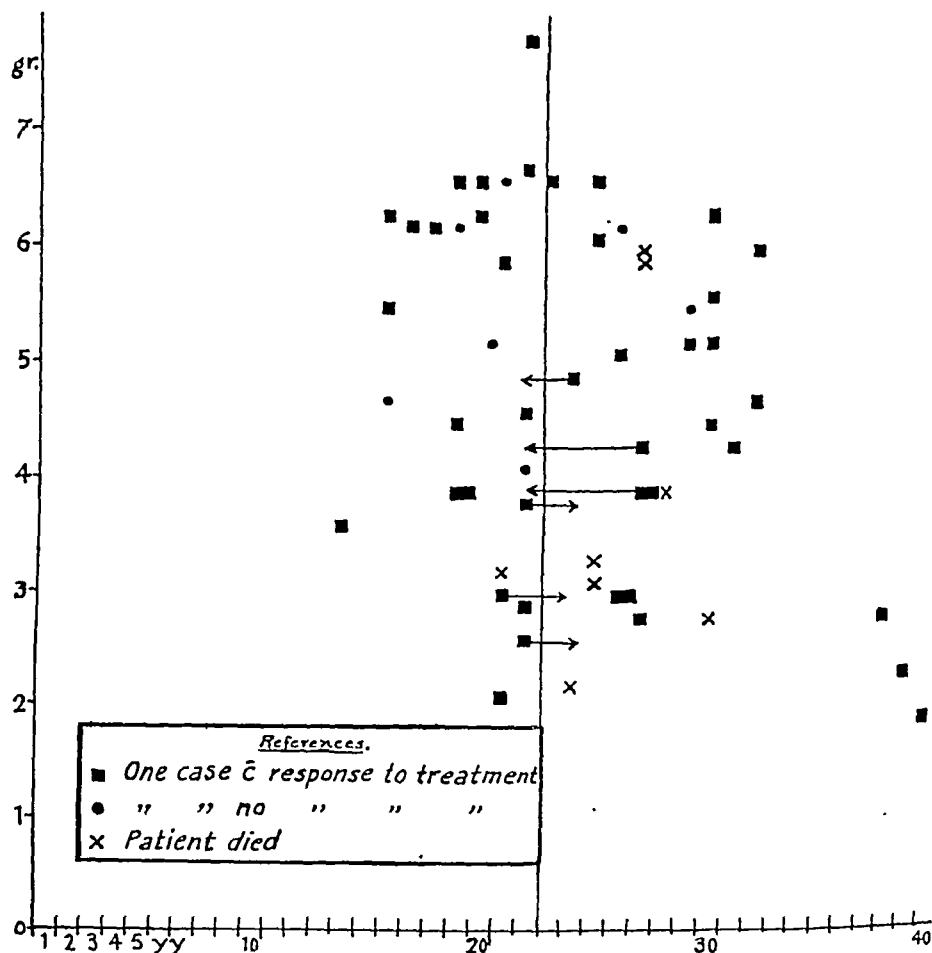
As we have stated above, in this series we followed the cases for some time and the blood picture naturally changed. In the successfully-treated cases there was a tendency, as one would expect, for the MCH and MCV to move towards normal, but some of the hypochromic microcytic cases remained as such, even after the hæmoglobin had increased to 'normal', or to such a level that the patient could be discharged from hospital and was thereby lost to us.

On the other hand, there was an early change in the picture in some cases, before treatment had been commenced or could possibly have taken effect, for example, case 49 who was originally hypochromic normocytic, with a few days of iron administration, though the hæmoglobin level fell, became orthochromic macrocytic. It was tempting to classify the cases from the beginning on their *general* picture and response to treatment, but as this method involved too many arbitrary decisions we decided to classify them at first on their initial count.

In Graph 1 we have plotted our cases according to the degree of anæmia and the hæmoglobin content of the corpuscles. Though they do not fall quite so easily into groups, as did the cases in the previous series, if we adopt the same criterion for hypochromicity, namely below 22 $\gamma\gamma$, and a slightly higher level than in the previous series for hyperchromicity, 32 $\gamma\gamma$, the cases seem to fall into three groups, the fourth group (of our previous series), the slightly-anæmic hyperchromic

group, having automatically disappeared in this series as the standard for anæmia was higher.

GRAPH 1.



Showing the hæmoglobin in grammes per 100 c.c. of blood and the mean corpuscular hæmoglobin of the cases in this series.

The line at 22 $\gamma\gamma$ divides the hypochromic from the orthochromic.

The arrows indicate the redistribution that we made on the response to treatment.

With reference to mean corpuscular volume, in view of the slight uncertainty about the measurements, referred to above, we have given this a secondary place, in the classification, but have adopted two arbitrary levels at 75 cu. μ and 105 cu. μ ; below the former, cells are considered to be microcytic, and above the latter, macrocytic, those between being considered normocytic.

Provisional classification.—The cases in this series thus fall into three groups as follows:—

A. Hypochromic.—There are in this group 26 cases: of these 7 are microcytic, 18 are normocytic and 1 macrocytic; of these 26 cases, (1) 16 (6 microcytic,

9 normocytic, 1 macrocytic) received iron only and responded to the treatment in varying degrees, (2) 5 (1 microcytic) had iron only and did not respond, (3) 4 (all normocytic) had mixed treatment and responded to treatment in varying degrees, and (4) 1 (normocytic) had mixed treatment and died.

B. Orthochromic.—This group consisted of 28 cases, (1) of these 18 (4 macrocytic and 14 normocytic) responded to treatment in varying degrees, (2) 7 died (6 normocytic and 1 macrocytic), and (3) 2 (both normocytic) did not respond to mixed treatment, and 1 received no treatment.

C. Hyperchromic.—The three hyperchromic cases were all macrocytic, and all responded to treatment with liver extract.

RESPONSE TO TREATMENT.

In all cases the treatment was given under the instruction of Dr. Fraser, Medical Officer In-Charge of the hospital and the group of gardens. The treatment given was that usually given in the hospital, but Dr. Fraser very kindly agreed to give one 'hæmatinic' at a time, so that the effect could be seen. He was naturally guided to some extent by our hæmatological findings, which of course were placed at his disposal, but as far as possible he met our request that either iron or Marmite should be given first, except in cases in which a liver preparation seemed to be indicated clearly, for example, in the cases of severe anæmia when it was known from our examination that this was not markedly microcytic, and in the macrocytic cases.

Iron was given in the form of ferrous sulphate tablets, in doses of ($6 \times 3 =$) 18 grains a day in most cases, but in a few ($6 \times 4 =$) 24 grains a day were given. (One tablet contained 6 grains of dry ferrous sulphate and a trace of copper and manganese.)

The usual dose of Marmite was 2 drachms three times a day: this was the maximum dose that the patients could be induced to take in most instances.

The only form of liver therapy employed was Campolon intramuscularly: the usual dose was 2 c.c. daily, and it was normally continued for 10 days in the first instance. In a few cases 4 c.c. were given daily at first, or for the whole 10 days, but in the following notes, unless otherwise stated, 2 c.c. was the daily dose.

Blood transfusion after 'grouping' the bloods of the patient and donor—always a close relative—was performed in five cases. It was usually adopted in extreme cases as a life-saving measure and always in conjunction with other treatment. No *specific* action of blood transfusion was noted, but it undoubtedly helped to tide some patients over a critical period. Of the transfused patients two died.

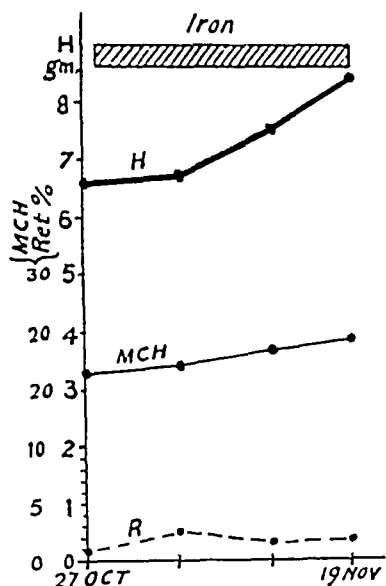
On tea-estate standards the hospital is a very good one, but of course there are no nurses and the patients depend for nursing and feeding on their relatives and friends, who are permitted to stay with them.

The diet itself is provided by the hospital and is a better one than the coolies normally take in their homes.

A 1. Hypochromic cases.—Of the 16 cases in which only iron was given and there was response to treatment, (a) 9 had a moderate anæmia and an initial

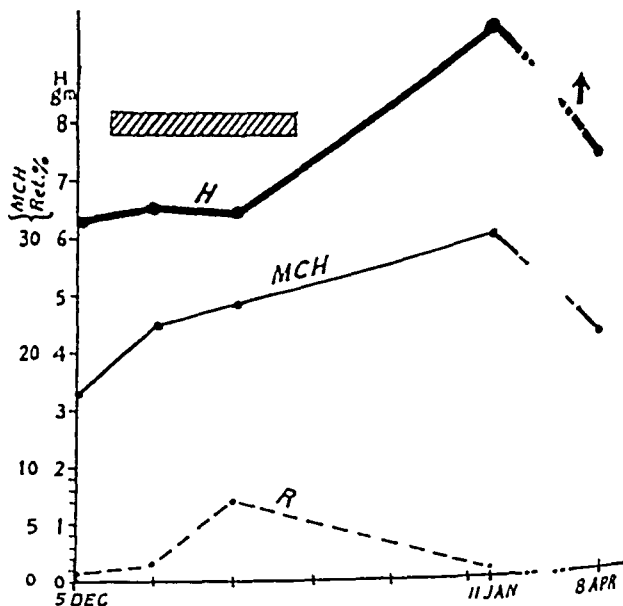
hæmoglobin level above 5.5 g. Of these (i) 8 were ante-partum (cases 9, 13, 22, 24, 25, 38, 41, and 54) and (ii) 1 (case 10) post-partum.

GRAPH 2.



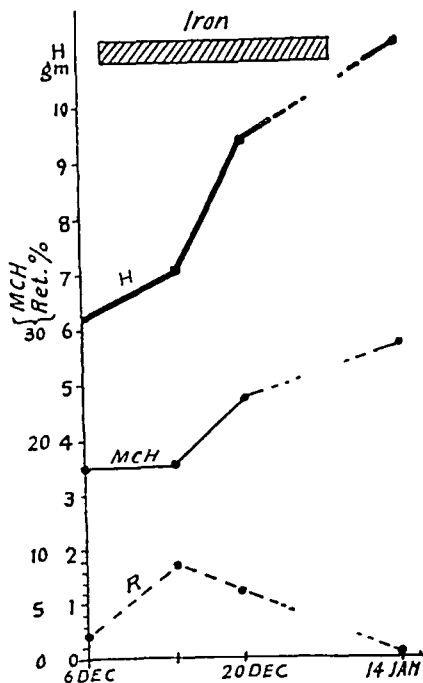
CASE 9.

GRAPH 3.



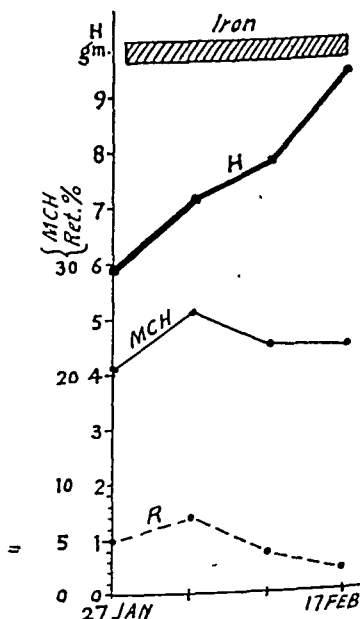
CASE 24.

GRAPH 4.



CASE 25.

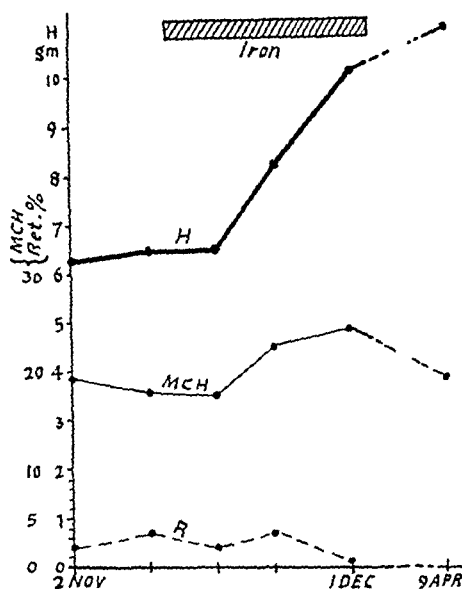
GRAPH 5.



CASE 41.

- (i) Three had a reticulocyte response of 7 per cent or more, two others a definite response of a lower grade, and three no response at all. In six the MCH rose to above 22 $\gamma\gamma$, but the other two remained hypochromic. The MCV rose in all, but only one microcytic case became macrocytic temporarily, and one normocytic became macrocytic at the time of discharge.
- (ii) The post-partum case (10) had no reticulocyte response, but the hæmoglobin rose to normal rapidly and the MCH and MCV increased to the orthochromic normocytic level. Four months later her hæmoglobin had increased further to 11.0 g., but she was again hypochromic and was now microcytic: she had a heavy hookworm infection.

GRAPH 6.



CASE 10.

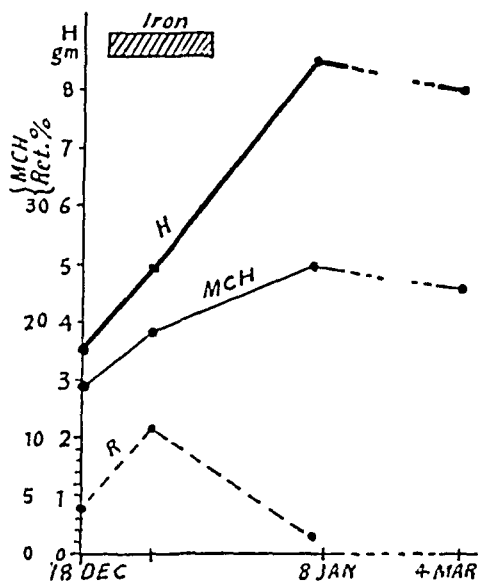
In the eight ante-partum cases of moderate anaemia the response to iron treatment was usually sub-maximal and without or with only a low reticulocytosis; the inhibitory effect of the foetus was apparent (see Graphs 2, 3, 4, and 5—cases 9, 24, 25, and 41, respectively). But in the post-partum case the progress was rapid and maintained despite a heavy hookworm infection, the effect of which is shown in the hypochromicity.

(b) Seven cases had more severe anaemia; (i) of these three were ante-partum, and (ii) four post-partum.

Case 26 (Graph 7).—A primipara in the third month of pregnancy ($H = 3.6$ g., $MCV = 71$, $MCH = 14.5$). Iron was given and there was a sharp reticulocyte response on the fifth day to 11 per cent; after three weeks of iron treatment the hæmoglobin had risen to 8.53 g., the MCV to 97 cu. μ and MCH

to 25.1 $\gamma\gamma$: she was then discharged and two months later the hæmoglobin had fallen slightly to 8.0 g. She had a heavy hookworm infection.

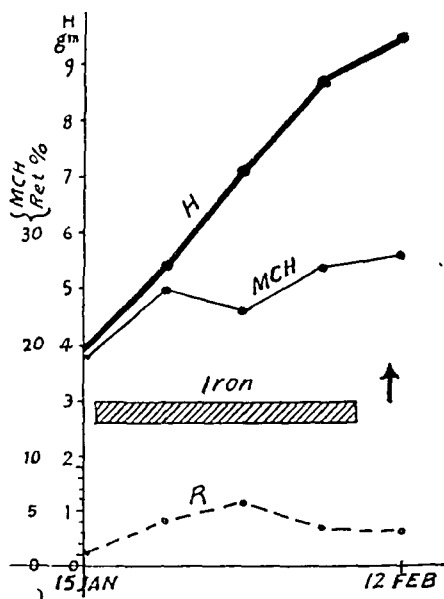
GRAPH 7.



CASE 26.

Case 35 (Graph 8).—A primipara ($H = 3.9$ g., $MCV = 86$, $MCH = 18.9$). She improved on iron treatment, but with little reticulocyte response. After delivery the improvement was maintained.

GRAPH 8.



CASE 35.

Arrow indicates delivery of a live child.

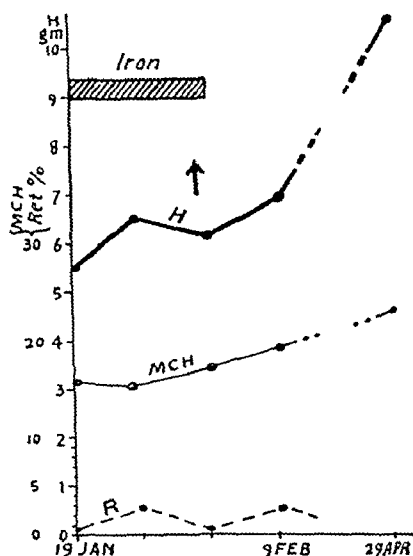
Case 37 (Graph 9) ($H = 5.5$ g., $MCV = 62$, $MCH = 15.6$).—She improved slightly when iron was given and the MCH increased but there was no reticulocyte response. After delivery there was further improvement and three months later the hæmoglobin was 10.6 g. and the MCH 23.2 $\gamma\gamma$.

These three are typical cases of iron-deficiency anæmia. In the first the fœtus was small and the response was maximal, but in the second the hæmoglobin and the mean corpuscular hæmoglobin increased, but without any reticulocyte response, and in the third case the limiting influence of the full-term fœtus was apparent and there was little improvement until after delivery.

(ii) *Post-partum*.—Three of these four cases came under observation one week after delivery.

Case 43 (Graph 10) ($H = 4.5$ g., $MCV = 89$, $MCH = 21.1$).—There was a distinct reticulocyte response (11.2 per cent on the fifth day) to iron and the hæmoglobin rose to 8.25 g. in three weeks, but there was no change in the MCH.

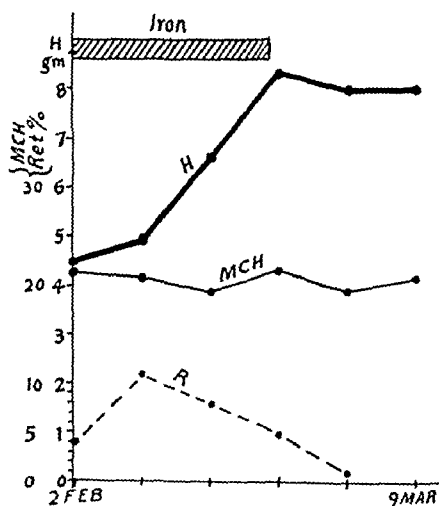
GRAPH 9.



CASE 37.

Arrow indicates delivery of a live child.

GRAPH 10.



CASE 43.

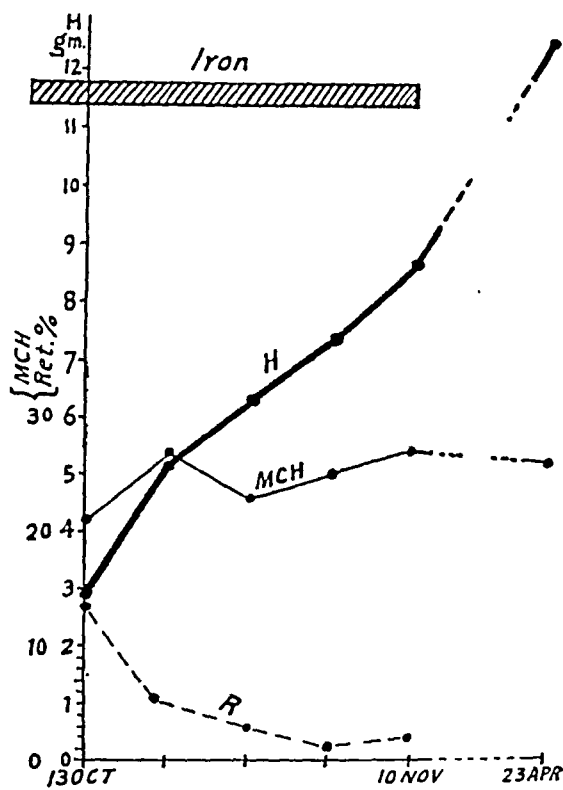
Case 53 ($H = 4.5$ g., $MCV = 75$, $MCH = 19.5$).—The hæmoglobin rose to 7.2 g. after about a month's iron treatment, but there was little reticulocyte response and the MCH rose only to 20.5 $\gamma\gamma$.

Case 56 ($H = 3.85$ g., $MCV = 81$, $MCH = 19.0$).—There was a sharp reticulocyte response (10.6 per cent on sixth day) to iron and a rise in hæmoglobin and MCH (to 25.2 $\gamma\gamma$) which was not maintained as there was evidence of sepsis, but later the hæmoglobin improved again.

The fourth patient (case 3—Graph 11) ($H = 2.9$ g., $MCV = 107$, $MCH = 21.1$) came under observation four days after abortion (sixth month), but she had been taking iron for five days.

The reticulocytes were 13.6 per cent. The hæmoglobin rose to 8.5 g. within a month. Four months later it was 12.5 g. and the MCV and MCH 89 cu. μ and 26.1 $\gamma\gamma$, respectively.

GRAPH 11.



CASE 3.

In the first three cases of this last group [A 1 (b)(ii)] the improvement appears to have been due to the iron and not to the delivery, but in the last it might have been due to either.

A 2. Hypochromic cases, failures, and deaths.—Of the former, four severe ante-partum cases (12, 27, 32, and 34) were all microcytic, or below 80 cu. μ , all were in the last trimester, and were under iron treatment for at least a month. None showed any response and all remained hypochromic. None had any serious clinical complications to account for the failure of response to treatment.

One was hypochlorhydric, one showed normal gastric acidity, and one was hyperchlorhydric. The indirect van den Bergh was negative in all four. All showed a slight reticulocytosis at one time or another. Two had palpable spleens. All had a hookworm infection, but none a heavy one.

Three had uncomplicated labours and subsequent examinations showed either a lower level of hæmoglobin or no change.

One failure was a two-months' post-partum (five-months' abortion) (case 33). She was a normocytic case at first, but later the cell became microcytic. She received iron for about a month without improvement. She was febrile and a purulent discharge was coming from the uterus: carcinoma was suspected.

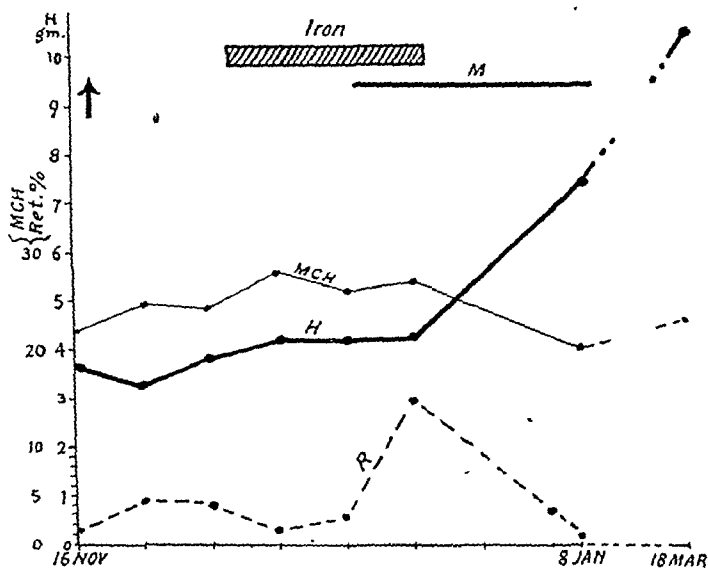
The details of the patient who died are given below:—

Case 49.—Post-partum ($H = 3.0$ g., $MCV = 82$, $MCH = 20.7$). Later, the MCV rose to 122 cu μ . There was no response to iron, but to Campolon, two courses of 10 doses each of 2 c.cm., there was a slight reticulocyte response but no improvement in the red count until a little time before death when it rose in a most unaccountable manner. She had chronic intractable dysentery which showed no signs of improvement even to the end.

A 3. *Hypochromic cases with mixed treatment.*—There were four in this group.

Case 20 (Graph 12).—Ante-partum ($H = 3.7$ g., $MCV = 83$, $MCH = 21.8$). Delivery occurred the day after admission. Fourteen days of iron made no appreciable difference to the hæmoglobin, but the MCH and MCV rose. Then Marmite was given, and there was a sharp reticulocytosis to 15 per cent, a steady rise in hæmoglobin from 4.3 to 7.4 in 14 days, and a fall in MCH to 20 $\gamma\gamma$ again. Improvement continued after discharge.

GRAPH 12.



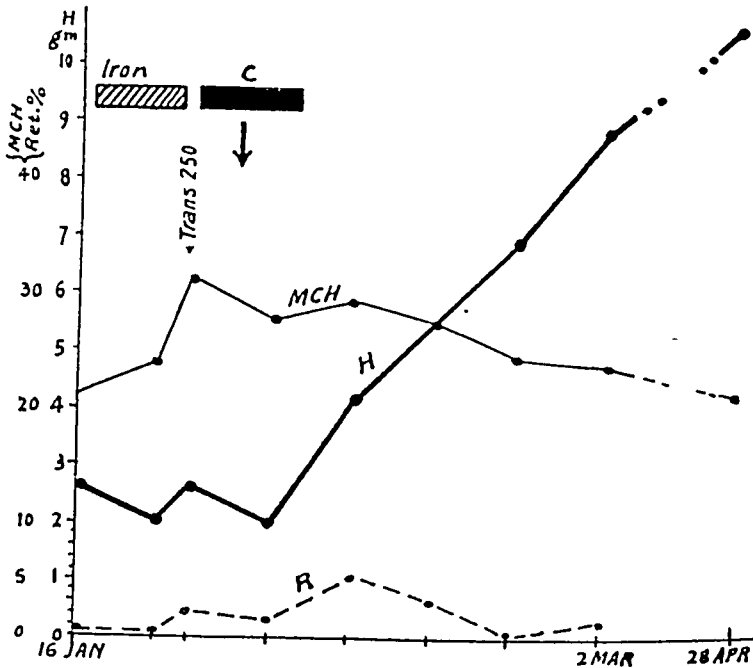
CASE 20.

Arrow indicates delivery of a live child. M = Marmite.

Case 36 (Graph 13).—Ante-partum ($H = 2.6$ g., $MCV = 84$, $MCH = 21.2$). Iron only led to a rise in MCH and MCV to the macrocytic level, but no increase in hæmoglobin. A small blood transfusion was given and then Campolon, four days after which abortion (fifth month) occurred. There was no immediate response but 10 days later there was a reticulocyte rise to 5.5 per cent, and

a sharp haemoglobin rise which continued until it reached 10.7 g. three months later: the MCV and MCH meanwhile fell again.

GRAPH 13.

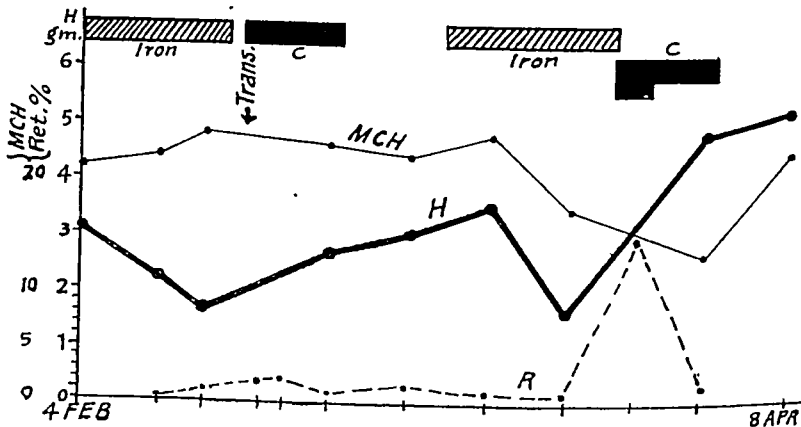


CASE 36.

Arrow indicates abortion.

Case 44 (Graph 14).—Post-partum (H = 3.0 g., MCV = 90, MCH = 20.9). Delivery had occurred a week before admission. Iron for 12 days had no effect. Transfusion and Campolon for 10 days produced a slight recovery in haemoglobin and the appearance of a few reticulocytes where there had been none before. Iron again was followed by a fall in haemoglobin and MCH. Campolon 5 c.c. for three days and 2 c.c. for seven days produced a sharp reticulocyte rise to 15 per cent on the fourth day and a steady rise in haemoglobin.

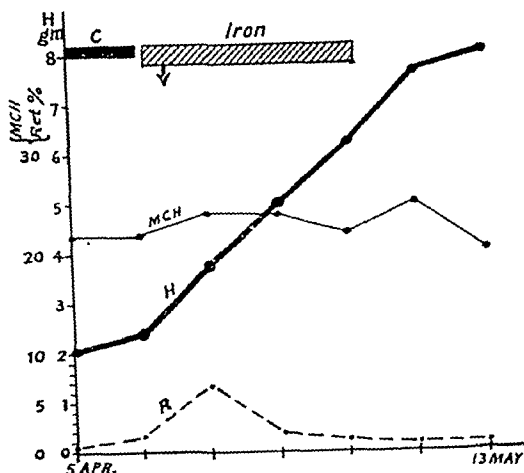
GRAPH 14.



CASE 44.

Case 52 (Graph 15).—Ante-partum ($H \approx 2.1$ g., $MCV = 104$, $MCH = 21.7$). There was no response to Campolon for eight days, but two days after iron was commenced delivery occurred. On the seventh day of iron the reticulocytes had risen to 6.5 per cent and the hæmoglobin rose steadily.

GRAPH 15.



CASE 52.

Arrow indicates delivery of a dead child.

Thus, of the four cases, case 20 undoubtedly responded to Marmite, cases 36 and 52 (Graphs 13 and 15) improved after delivery, in one Campolon and in the other iron being the principal hæmatinic factor, and in case 44 (Graph 14) a febrile attack appears to have prevented improvement, but eventually large doses of Campolon determined the hæmatological and clinical improvement.

Discussion on the hypochromic group.—Thus, in 16 cases in which iron only was given, there is evidence that they were all suffering from iron deficiency and in most of them the response was such that it pointed to iron as the main deficiency. Only in early pregnancy (case 26) was the response to iron maximal and comparable to that we have usually seen in the non-pregnant iron-deficient coolies. The presence of a well-developed fœtus seemed to slow down the response to treatment in nearly all cases, and when iron was given late in pregnancy, that is, immediately before delivery, improvement was often delayed until the uterus was empty.

In the hypochromic group, there were two cases in which the evidence points to Campolon and one in which it points to Marmite as the 'hæmatinics' determining improvement. But all these were only just in the hypochromic class at the first examination and on subsequent examinations they would have been classed as orthochromic, normocytic or even macrocytic.

Four of the 'failures' received iron only and therefore one cannot say definitely that they would not have improved on Campolon administration, but they were all very definitely and persistently hypochromic and often microcytic. and it seems very improbable that they would have improved on liver therapy. The other failure and the patient that died had complications.

B 1. Orthochromic group.—Out of the 18 cases in this group in which there was response to treatment, four were macrocytic and 14 normocytic; three cases were post-partum and 15 ante-partum, but in 10 of the latter delivery occurred whilst the patient was under observation.

In nearly all these cases more than one hæmatinic was given, and in the great majority either iron or Marmite was given for a time and the liver treatment reserved for when they did not respond or responded slowly to these medicaments.

These 18 cases can be divided according to the main factor in the response to treatment as follows:—

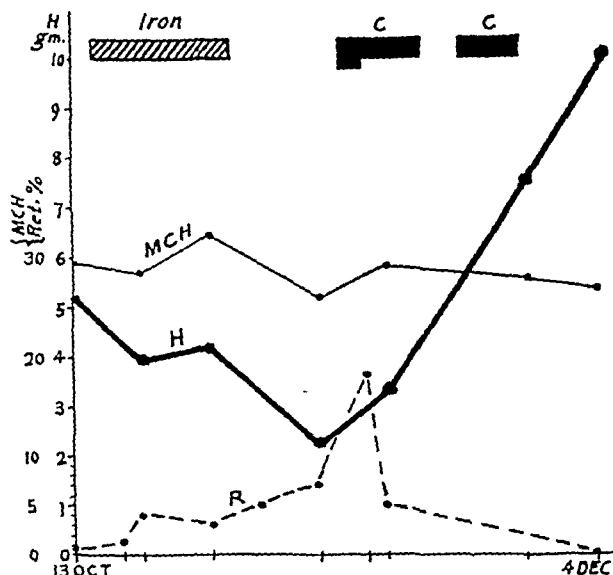
	Macrocytic.	Normocytic.	Ante-partum.	A. and P. partum.	Post-partum.
(a) Campolon, 8 cases ..	3	5	2	4	2
(b) Marmite, 5 „ ..	0	5	3	2	..
(c) Iron, 3 „	3	..	2	1
(d) Doubtful, 2 „ ..	1	1	..	2	..
TOTAL .. 18 „ ..	4	14	5	10	3

Liver treatment (Campolon).—This was given in adequate doses in 12 cases—(a)—8, (b)—1, (c)—2, and (d)—1. In the following eight cases it appeared to have a definite beneficial effect:—

Case 1 (Graph 16).—Post-partum (H = 5.2 g., MCV = 86, MCH = 29.5). Iron for two weeks caused a rise in the MCH to 32 γγ, a slight reticulocyte response, but a fall in hæmoglobin: with Marmite for 12 days the hæmoglobin fell further to 2.2 g. and the MCH fell to 26 γγ: Campolon (3 c.c.

$\times 2 + 2 \text{ c.c.} \times 15$) was followed by a sharp reticulocyte reaction of 18 per cent on the fourth day and a rapid rise of haemoglobin to 10 g. in 28 days.

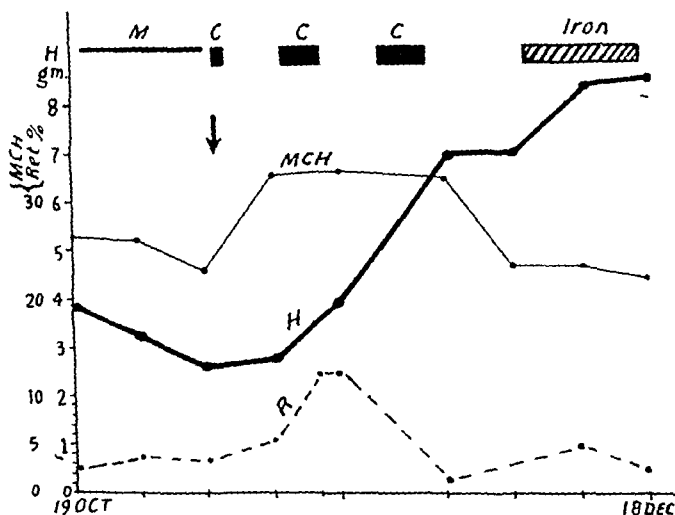
GRAPH 16.



CASE 1.

Case 7 (Graph 17).—Ante-partum (H = 3.85 g., MCV = 102, MCH = 26.7). Marmite was given for a fortnight and then a dose of Campolon after which abortion occurred, meanwhile the

GRAPH 17.



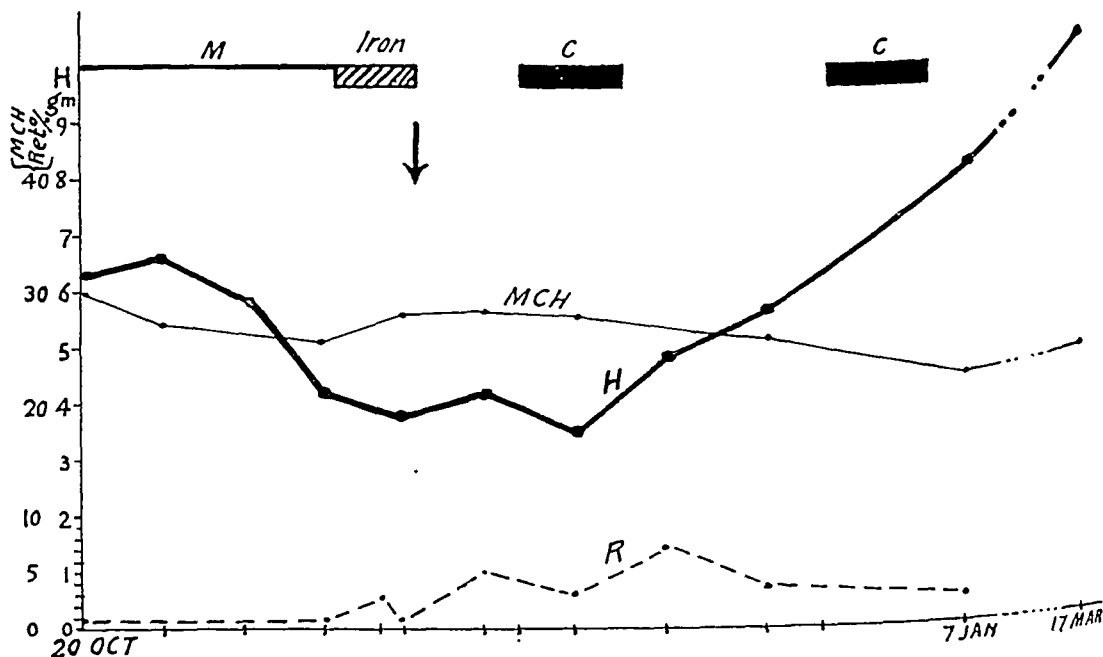
CASE 7.

Arrow indicates abortion.

hæmoglobin had fallen to 2.6 g. No treatment was given for a week during which period the hæmoglobin remained stationary: then five doses of Campolon were given and on the fifth day there was a reticulocyte response of 12.5 per cent and a sharp rise in hæmoglobin which was followed by a further rise to 7.0 g. eleven days later, after another five injections of Campolon. The hæmoglobin then remained stationary for a week and iron was given. This was followed by a further rise in hæmoglobin and a slight reticulocyte response to 5 per cent. The MCH rose after delivery to 33 $\gamma\gamma$ but fell to 23 $\gamma\gamma$ after the second course of liver.

Case 8 (Graph 18).—Ante-partum (H = 6.3 g., MCV = 105, MCH = 29.8). Marmite for three weeks and iron for one week caused no reticulocyte response or rise of hæmoglobin which was now down to 3.8 g. Abortion occurred followed by sepsis and fever and a further fall to 3.4 g. Campolon was given for 10 days and again after an interval of another 10 days: there was a delayed rise after the first course which was maintained until 8.2 g. was reached on discharge. Two and a half months later the hæmoglobin had reached 10.3 g. The reticulocyte count was irregular, but the MCH fell on Marmite administration, rose again when iron was given, fell to below 22 $\gamma\gamma$ during the liver administration and finally rose to 24 $\gamma\gamma$ when the hæmoglobin had reached 10 grammes.

GRAPH 18.

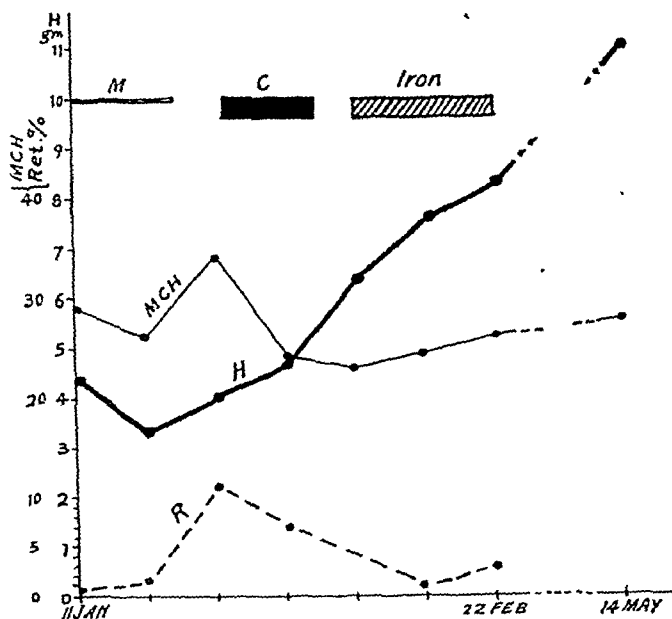


CASE 8.

Arrow indicates abortion.

Case 30 (Graph 19).—Ante-partum (H = 4.53 g., MCV = 106, MCH = 29.2). Marmite was given for 16 days: there was first a fall to 3.3 g. and then a recovery in the hæmoglobin accompanied by a sharp reticulocyte response, 11 per cent. However, as the cell was definitely macrocytic (130 cu. μ and 34.4 $\gamma\gamma$) Campolon was given for 10 days. The hæmoglobin curve took a sharp upward turn but there was no further reticulocyte rise. Iron was given now as the cell size had fallen, but there was little evidence that this had any effect though there was a reappearance of reticulocytes. The patient was discharged at 8.3 g., and three months later was 11 g., parturition having occurred meanwhile.

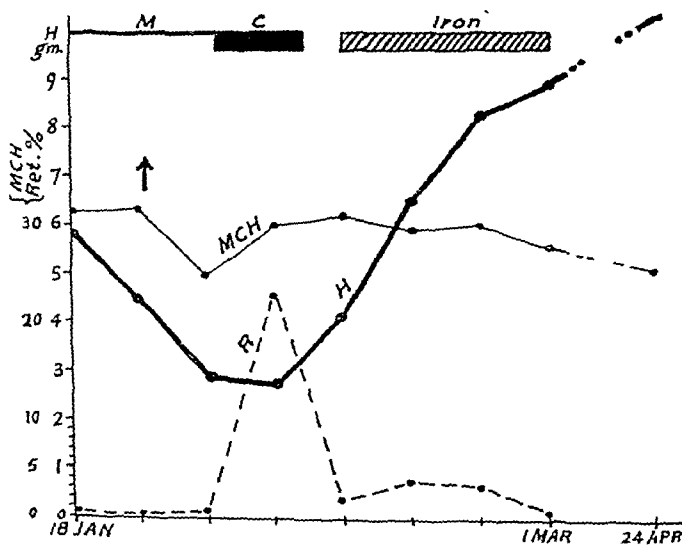
GRAPH 19.



CASE 30.

Case 31 (Graph 20).—Ante-partum ($H = 5.9$ g., $MCV = 103$, $MCH = 31.7$). The hæmoglobin fell to 2.75 g. after 15 days' Marmite administration during which time delivery occurred. A week

GRAPH 20.



CASE 31.

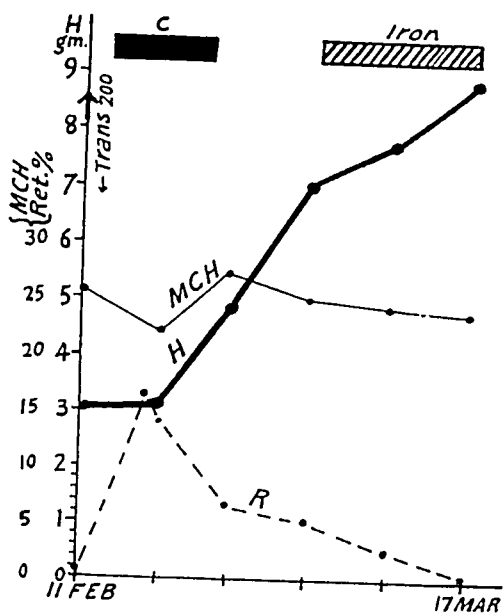
Arrow indicates delivery of a live child.

after delivery Campolon was given for 10 days: on the sixth day there was a sharp reticulocyte reaction and from this time the haemoglobin rose by 5.55 g. in three weeks to 8.3 g. and eventually to 10.5 g. Iron was given for three weeks; it had no effect on the haemoglobin curve, but caused a slight rise in reticulocytes to 4 per cent.

Case 46 (Graph 21).—Post-partum ($H = 3.0$ g., $MCV = 102$, $MCH = 25.6$). Admitted immediately after delivery, after which a small transfusion (200 c.c.) was given. Campolon (2 c.c.) was given for 10 days. This produced a reticulocyte response of 16 per cent on the fifth day, but no immediate rise in haemoglobin: there was a sharp rise to 4.8 g. on the twelfth day and to 7.0 g. on the nineteenth. Iron was given for 14 days during which the haemoglobin continued to rise, but there was no reticulocyte response.

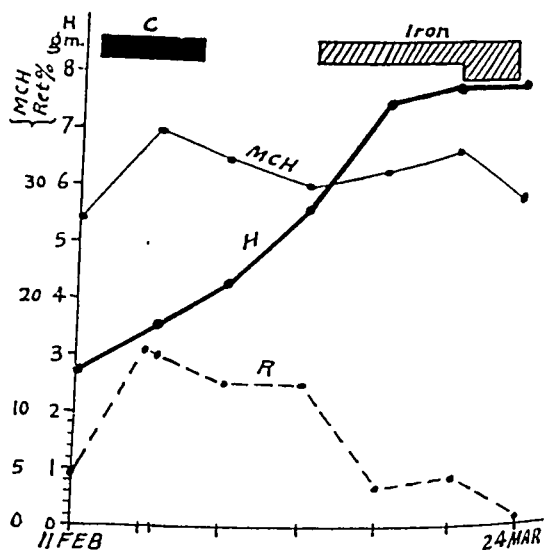
Case 47 (Graph 22).—Ante-partum sixth month of pregnancy ($H = 2.75$ g., $MCV = 107$, $MCH = 27.0$). Campolon was given for 10 days: this was followed by a sharp reticulocyte reaction of 16 per cent and a steady rise in haemoglobin to 7.6 g. in four weeks. Despite three weeks of iron treatment no further appreciable rise occurred.

GRAPH 21.



CASE 46.

GRAPH 22.



CASE 47.

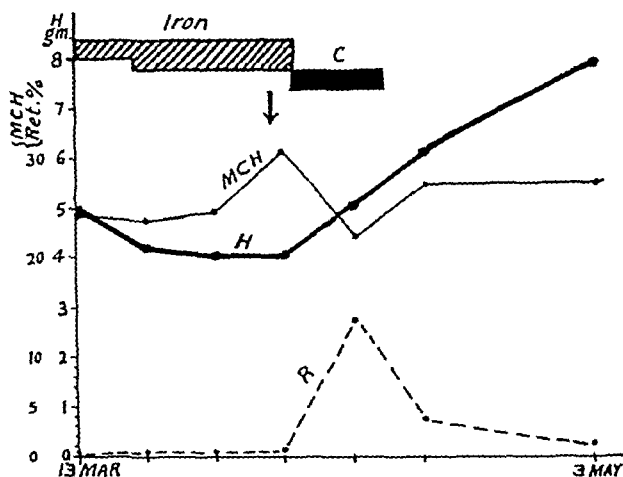
Case 50 (Graph 23).—Ante-partum ($H = 5.1$ g., $MCV = 92$, $MCH = 25.3$). The haemoglobin fell to 4.1 g. in three weeks with iron treatment. Parturition then occurred. Two days later a 10-day course of Campolon was started: on the sixth day of this there was a sharp reticulocytosis of 14 per cent and the haemoglobin started to rise, reaching 8 grammes in 30 days from the commencement of the Campolon administration.

Again there seems little doubt of the sub-maximal effect of Marmite, the greater effect of Campolon, and the doubtful subsidiary effect of iron.

Iron was given in all these eight cases: it had a possible slight effect in three and none in the rest. Marmite was given in five cases and had a possible slight effect in two but none in the rest.

Marmite.—In the following five cases Marmite appears to have been the main factor in determining improvement.

GRAPH 23.

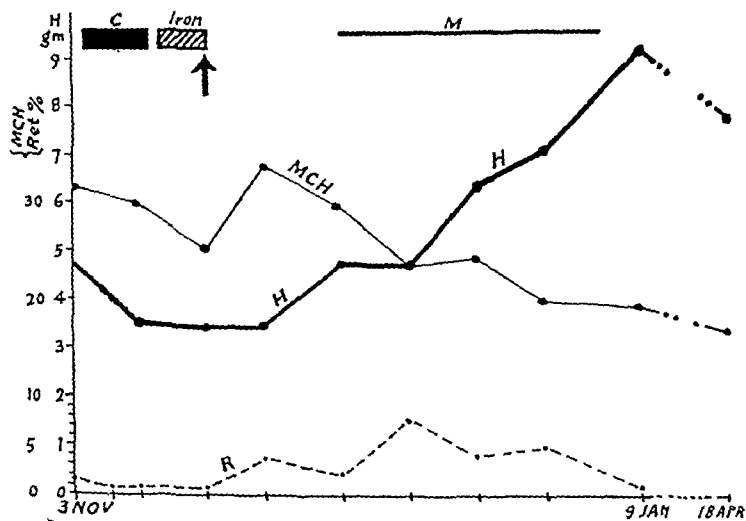


CASE 50.

Arrow indicates delivery of a dead child.

Case 11 (Graph 24).—Ante-partum ($H = 4.68$ g., $MCV = 103$, $MCH = 31.6$). Before delivery Campolon was given for eight days and iron for six: there was a fall in haemoglobin to 3.5 g.; at this level it remained for a fortnight in the middle of which time she was delivered of a live baby.

GRAPH 24.



CASE 11.

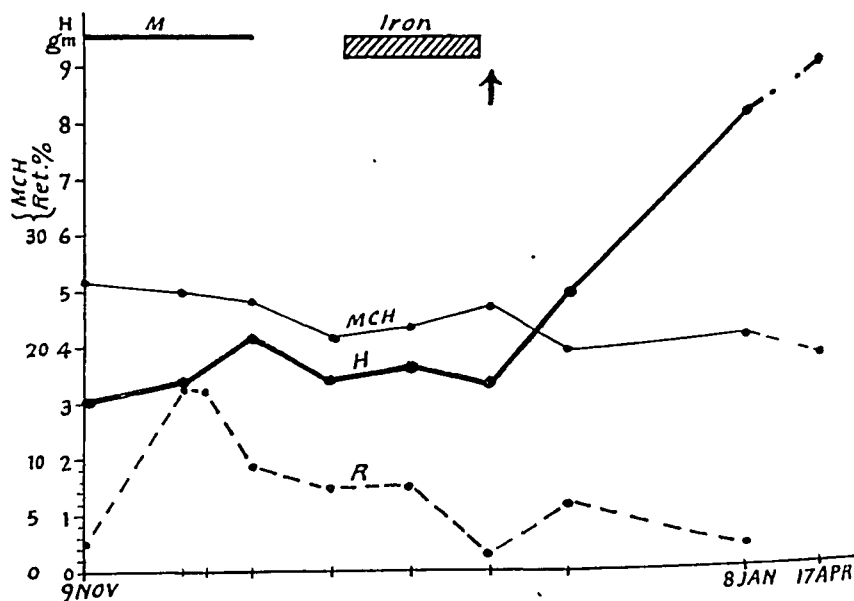
Arrow indicates delivery of a live child.

Marmite was then given for a month and there was a sharp reticulocyte rise (7.5 per cent) and a steady increase in hæmoglobin to 9.2 g. Three months later the hæmoglobin had fallen to just below 8 grammes. The size of the cell which at the time of delivery reached the macrocytic hyperchromic level fell steadily and became microcytic hypochromic.

In this case the inhibitory effect of the foetus prevented the liver and/or iron from taking effect, and Marmite undoubtedly accelerated the recovery, but this was not maintained on account of an underlying iron deficiency. The patient had a heavy hookworm infection.

Case 19 (Graph 25).—Ante-partum (H = 3.0 g., MCV = 103, MCH = 25.9). Marmite was given for 14 days: there was a sharp reticulocyte response of 16 per cent on the eighth day, an improvement in the hæmoglobin to 4.2 g., and a fall in the MCV and in the MCH which eventually reached 21 $\gamma\gamma$. The hæmoglobin fell when Marmite was discontinued. Iron for 12 days had no effect, but then parturition occurred. There was a slight reticulocyte rise seven days later and a steady rise in hæmoglobin to 8.2 grammes within three weeks. Three months later it was 9.0 g.

GRAPH 25.



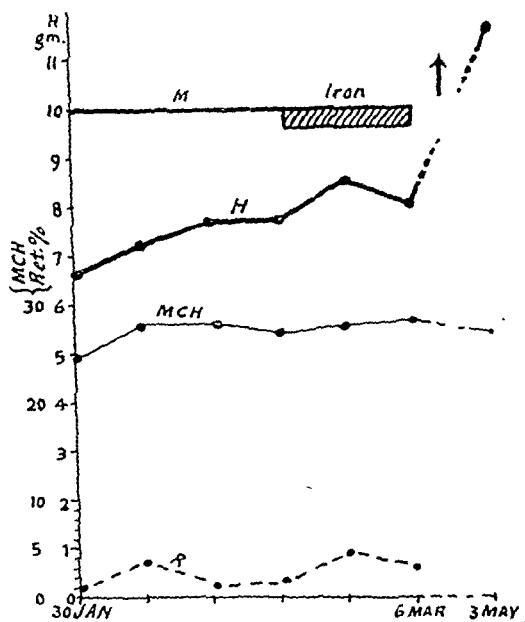
CASE 19.

Arrow indicates delivery of a live child.

The effect of Marmite in this case seems not to have been great and only temporary, but to have been definite, as it was threefold on the hæmoglobin, on the size of the cell and on the reticulocytes. After the Marmite had been taken the anaemia was hypochromic throughout, except on the day of delivery: the inhibitory influence of the foetus was clearly shown in this case.

Case 42 (Graph 26).—Ante-partum ($H = 6.6$ g., $MCV = 85$, $MCH = 24.4$). Marmite produced a slight reticulocyte response, 4 per cent, on the seventh day and a rise of hæmoglobin to 7.7 g. in a fortnight, but there was no further rise during the next week and iron was substituted: there was another slight reticulocyte response, 4.5 per cent, and a slight further rise of hæmoglobin to 8.5 g., but this rise was not maintained. Two months later (delivery having occurred in the interval) the hæmoglobin was 11.6 g., a high figure for her class.

GRAPH 26.



CASE 42.

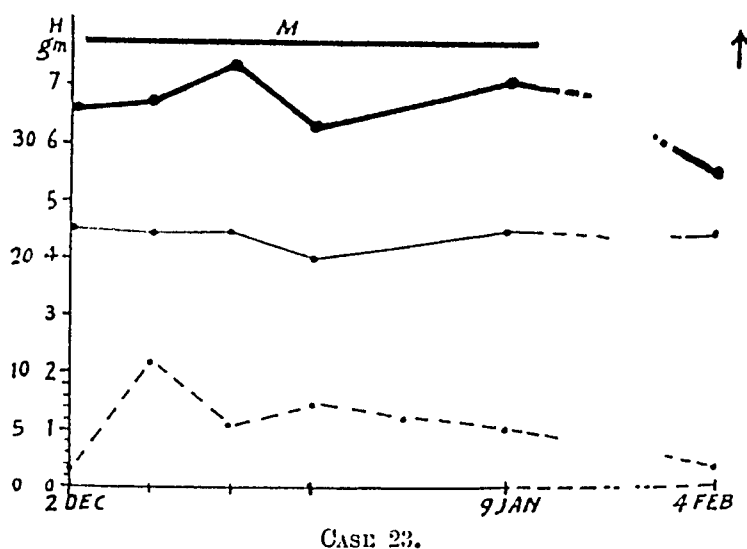
Arrow indicates delivery of a live child.

Again the inhibitory effect of the foetus is evident: the effect of Marmite was small but undoubted, and there is a suggestion that iron was also effective.

Case 23 (Graph 27).—Ante-partum throughout observation ($H = 6.6$ g., $MCV = 85$, $MCH = 22.8$). Marmite was given for six weeks. There was a sharp reticulocytosis (11 per cent) on the seventh day, and a rise in hæmoglobin to 7.4 g., but this level was not maintained and at the end of treatment was only 7.2 g. After discontinuance of the Marmite the level fell to 5.5 g. just before delivery. The MCH was almost constant throughout but once fell into the hypochromic class.

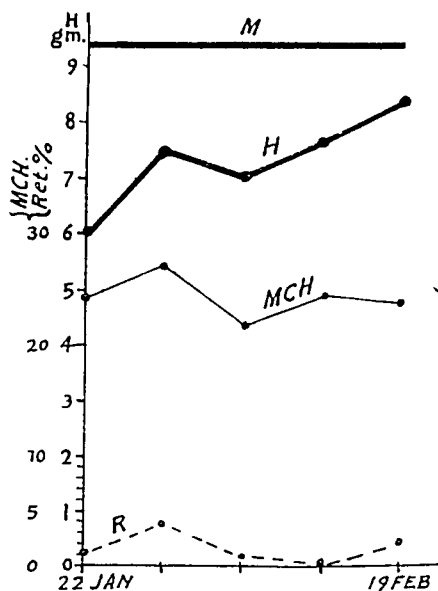
The undoubted but weak action of Marmite in the presence of the foetus is shown here. In view of the small size of the cell it seems probable that iron deficiency was an important factor.

GRAPH 27.



Case 40 (Graph 28).—Ante-partum throughout observation ($H = 6.0$ g., $MCV = 96$, $MCH = 24.2$). Marmite only was given for four weeks: improvement was slow but steady, the final reading being 8.4 g.; there was a slight reticulocyte response on the seventh day.

GRAPH 28.



In these five ante-partum cases the undoubted but sub-maximal effect of Marmite in the presence of a well-developed foetus, and in case 11 the rapid

improvement when the uterus had been emptied are clearly shown. In two there is also evidence of response to iron as well.

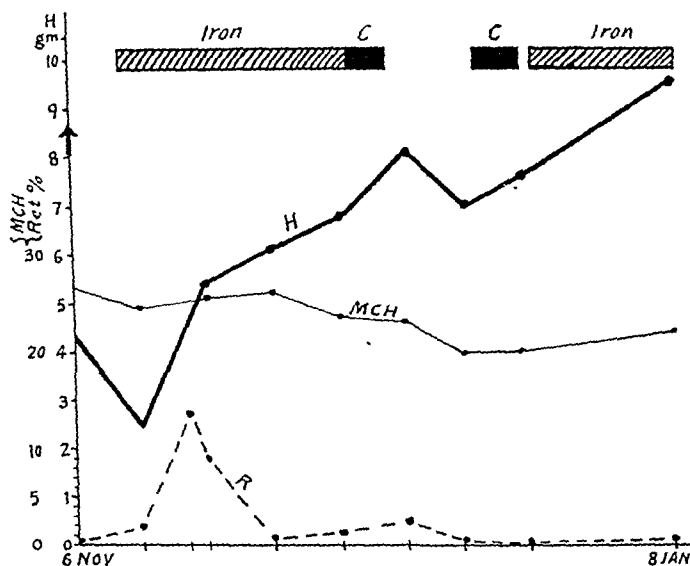
Iron.—There were three cases in which iron appears to have been the important factor in determining improvement.

Case 6.—Ante-partum ($H = 3.85$ g., $MCV = 97$, $MCH = 26.4$). Iron was given for eight days before and 21 days after parturition. There was a reticulocyte rise to 8 per cent on the seventh day of the second course. The MCH fell to 17.3 after parturition, but later recovered to 22 $\gamma\gamma$. After the reticulocyte rise the hæmoglobin rose to 5.5 g. but would go no higher until Marmite was given for three weeks when it rose further to 6.4 g. and the MCH again fell to 20 $\gamma\gamma$. Three months later the hæmoglobin was 6.3 g. and MCH 19 $\gamma\gamma$.

This was apparently a hypochromic iron-deficiency anæmia disguised by an ante-partum rise in the MCH; she had a very heavy hookworm infection (34,400 eggs per gramme of stool) which probably persisted and maintained the anæmia. There was slight evidence of the effect of Marmite.

Case 16 (Graph 29).—Post-partum ($H = 4.26$ g., $MCV = 82$, $MCH = 26.8$). There was a further fall of hæmoglobin to 2.48 g., possibly due to post-partum hæmorrhage.

GRAPH 29.

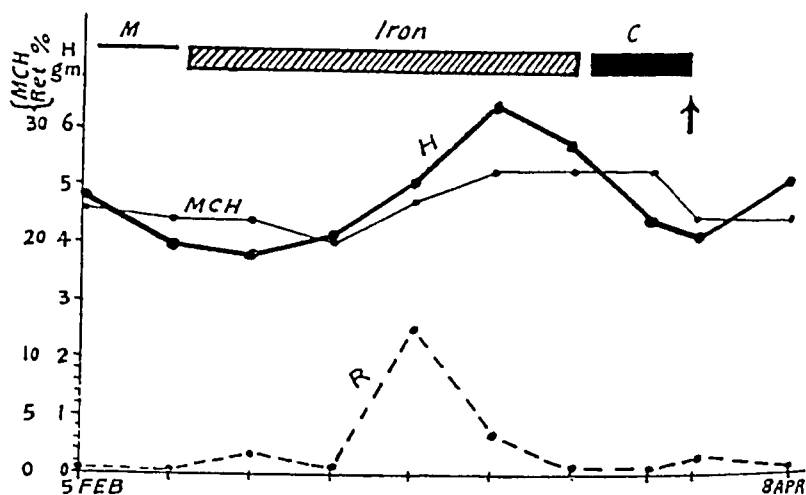


CASE 16.

Iron was given for three weeks, on the seventh day there was a sharp reticulocyte response to 14 per cent accompanied by a rapid rise in hæmoglobin to 6.8 g. in three weeks. Campolon was given, 10 doses with an interval of a week in the middle; this resulted in a net increase to 7.7 g. of hæmoglobin, but a distinct fall in the MCH to the hypochromic level. Further iron administration led to an increase to 9.6 g. and a slight rise in corpuscular hæmoglobin. This is again probably a hypochromic anæmia masked by a slight degree of Marmite-liver-factor-deficiency.

Case 45 (Graph 30).—Ante-partum ($H = 4.8$ g., $MCV = 85$, $MCH = 23.3$). During a week's treatment with Marmite the hæmoglobin fell to 4.0 g. and the MCH slightly to 22 $\gamma\gamma$. Iron was given for five weeks. There was no response for the first two weeks but during the third there was a sharp reticulocyte response to 12.5 per cent and the hæmoglobin commenced to rise, reaching 6.5 g. at the end of the fourth week of iron. The MCH also rose to 26.5 $\gamma\gamma$. After this the hæmoglobin fell again up to the time of delivery and 10 days of Campolon during this time had no effect. In the first week after delivery the hæmoglobin rose by a gramme.

GRAPH 30.



CASE 45.

Arrow indicates delivery of a live child.

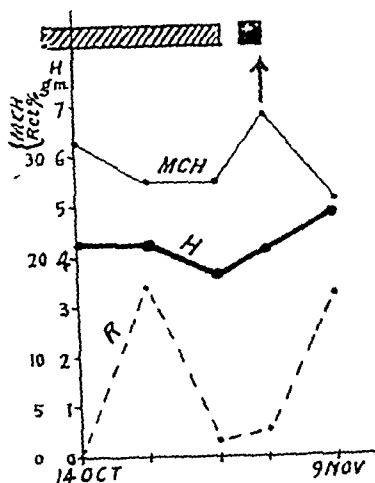
This was evidently a case of iron-deficiency anaemia in which the effect of iron was delayed and limited by the presence of a full-term foetus. Also it is really a hypochromic case which chance had placed in the orthochromic class.

Doubtful cases.—These were two cases in which it seems doubtful what was the cause of the improvement, but in both delivery played some part.

Case 5 (Graph 31).—Ante-partum ($H = 4.3$ g., $MCV = 115$, $MCH = 31.3$). Iron was given for 18 days and on the tenth day there was a sharp reticulocyte response to 17 per cent, but no increase in hæmoglobin until after parturition when there was another sharp reticulocyte rise to 16 per cent, an increase in hæmoglobin to 5.0 g. and a fall in MCH to 25.5 $\gamma\gamma$. Three days of Campolon were also given immediately before parturition.

There is evidence of iron deficiency, but other factors were too strong to allow the iron its full effect, these included a heavy hookworm infection. Whether the three Campolon injections produced the second reticulocytosis or whether this was due to the withdrawal of inhibition at parturition is not clear. It is a great pity the case was not followed longer.

GRAPH 31.

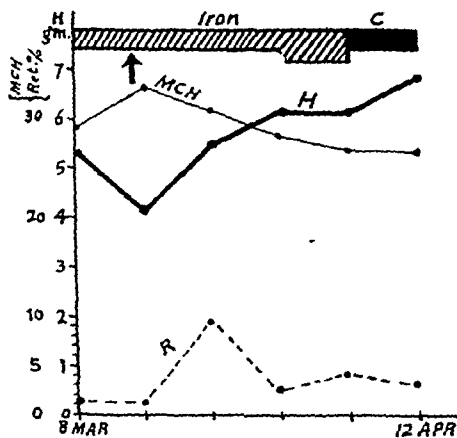


CASE 5.

Arrow indicates delivery of a live child.

Case 48 (Graph 32).—Ante-partum ($H = 5.2$ g., $MCV = 93$, $MCH = 28.9$). Iron was given for four weeks during which parturition occurred: there was a 10 per cent reticulocytosis 14 days after iron was commenced and seven days after parturition. The haemoglobin fell to 4.2 g. during parturition, then rose again to 6.2 g., but stopped there for a week: later Campolon was given for a week and there was a further rise of haemoglobin to 6.9 g., but no reticulocytosis.

GRAPH 32.



CASE 48.

Arrow indicates delivery of a live child.

This was probably a mixed deficiency which required more Campolon but the patient was not followed long enough.

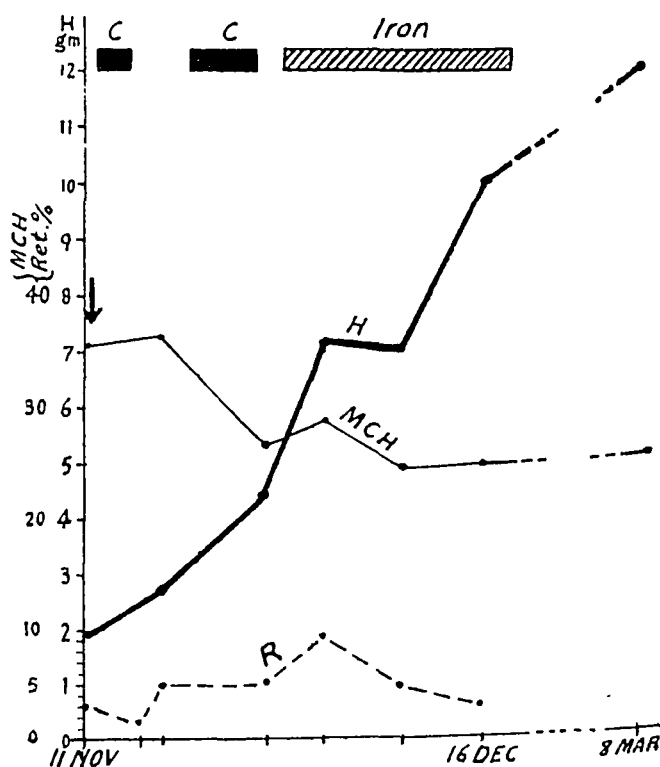
Thus, if we leave out of consideration the last two cases in which the interpretation seems very doubtful, of the 16 orthochromic cases in 13 the Marmite-Campolon factor appears to have determined improvement. The effect of Marmite by mouth is very clearly weaker than Campolon by the parenteral route and in the presence of the foetus its effect was sub-maximal. This inhibitory effect of the foetus was noticeable, though it was not so marked, even when Campolon was given, and the best responses occurred when the uterus was empty.

The other three cases in this orthochromic group showed evidence of iron being the main deficiency, but here again the inhibitory influence of the foetus was seen.

C. Hyperchromic group.—There are three cases in this group; all are macrocytic and all post-partum.

Case 17 (Graph 33).—Abortion (six months) immediately prior to admission ($H = 1.9$ g., $MCV = 158$, $MCH = 35$). Campolon was given on admission, 10 doses of 2 c.c. with an interval of four days in middle of course. The rise in haemoglobin was immediate and very rapid, but there was only a reticulocyte rise to 5 per cent. Iron given later seemed to cause a further rise in haemoglobin with a reticulocyte rise to 9 per cent. Meanwhile, the MCH fell to normal. The haemoglobin reached 10 g. in five weeks and three months later had reached 12 g.

GRAPH 33.

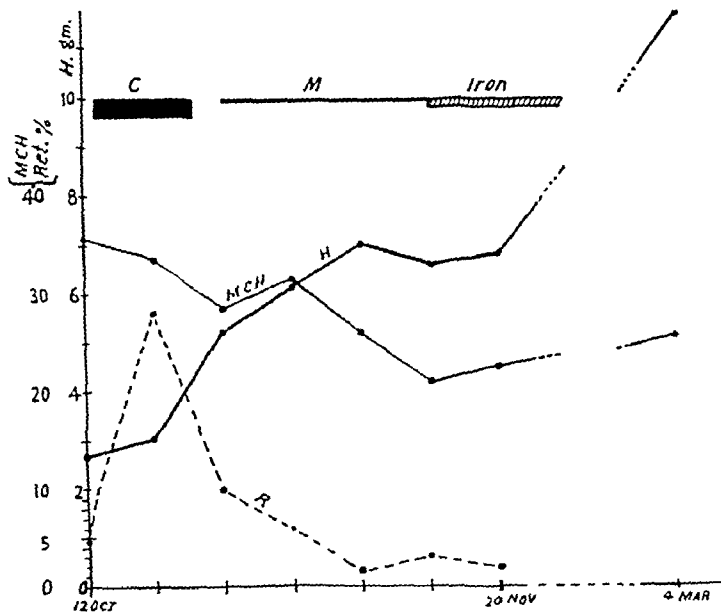


CASE 17.

Case 2 (Graph 34).—Immediately post-partum ($H = 2.75$ g., $MCV = 113$, $MCH = 35.7$), Campolon, 4 c.c. daily for 10 days, was given a few days after admission and there was a very sharp reticulocytosis (to 28 per cent) on the sixth day, and a rapid rise of haemoglobin. Marmite was then given but the rise was not maintained and meanwhile the MCH fell to 21.3 $\gamma\gamma$ and the MCV to 81 cu. μ .

Iron was then given and again the hæmoglobin rose but without any reticulocyte response. The MCH also rose to 25.4 $\gamma\gamma$.

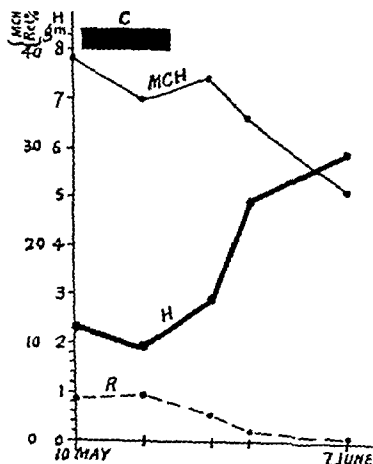
GRAPH 34.



CASE 2.

Case 55 (Graph 35).—Post-partum (six days) ($H = 2.3$ g., $MCV = 145$, $MCH = 39$). After a further slight fall in hæmoglobin, Campolon caused a steady rise of 4 g. in three weeks, but without appreciable reticulocyte response. The MCH also fell to 25.6 $\gamma\gamma$. The case was not followed further.

GRAPH 35.



CASE 55.

In each of these three cases there was a very rapid response to Campolon in the absence of any inhibiting factor.

In the first case (17) (Graph 33) also there is a suggestion that iron helped the improvement.

The second case (2) was an excellent example of mixed deficiency, an underlying iron deficiency being entirely clouded by the more important Marmite-liver-factor deficiency and there being a reaction to each hæmatinic in turn. She had hypochlorhydria and a heavy hookworm infection.

Deaths.—In the orthochromic series seven patients died. Of these, six died immediately after parturition: the last hæmoglobin estimations in these six cases were 1.9 g., 2.75 g., 2.75 g., 2.75 g., 3.85 g., and 5.9 g., respectively. Three patients, cases 4, 21, and 51, had one examination only. The other three were in hospital between a fortnight and three weeks: two received iron and one Marmite: of the former, one (case 57) had a transfusion also, and the other (case 18) Atebrin for two days as malignant tertian parasites were found (the indirect van den Bergh was \pm). The Marmite case (28), which was macrocytic, had a sharp reticulocyte response, 14.5 per cent on the thirteenth day, and the MCV and MCH fell sharply.

The seventh patient, case 29 ($H = 2.2$ g., $MCV = 98$, $MCH = 23.2$), had been delivered of a still-born child at the seventh-eighth month, two months previously, and had been anæmic since. She had Campolon, three doses, 5 c.c. and 2 c.c., and iron for 12 days, Marmite for six days and two blood transfusions but there was no response and she died immediately after the second transfusion. Latterly, the anæmia was macrocytic and the hæmoglobin was 2.3 g. at the last examination.

The failures.—In two orthochromic cases there was no response to treatment.

Case 15.—Ante-partum ($H = 5.4$ g., $MCV = 84$, $MCH = 23.5$). Marmite for three weeks resulted in a slight fall in hæmoglobin and a distinct fall in MCH to below 20 $\gamma\gamma$. Iron was given for three weeks: this caused a slight reticulocyte response to 6.8 per cent, a rise in hæmoglobin by 1 gramme to the original level, and a slight rise in MCH, but improvement was only temporary. Finally, Campolon was given for 10 days, and on the sixth day delivery occurred but there was no improvement and the patient was allowed to go home with hæmoglobin of 4.0 g., $MCV = 71.4$ cu. μ , $MCH = 17.4$ $\gamma\gamma$, now a definitely hypochromic microcytic case.

There was nothing in the patient's clinical condition to account for the failure: she had no splenic enlargement and only a low hookworm egg count.

Case 39.—Ante-partum ($H = 6.2$ g., $MCV = 103$, $MCH = 26.0$). Marmite was given for six days before and nine days after parturition without effect: the hæmoglobin had fallen to 4.3 g. a month after delivery.

Sepsis and pyelitis were the limiting factors in this case.

In one orthochromic normocytic case (58) no treatment was given during observation.

RECONSIDERATION OF CLASSIFICATION.

The successfully treated cases.—Reviewing the results of treatment generally, let us first consider whether we can improve on our classification of cases on

a treatment basis. A weakness in any argument that we base on our results is the fact that Campolon was only given in a very few of the hypochromic cases. We know from experience that in the definitely hypochromic case of anæmia Campolon is seldom of much value, but we are by no means satisfied that in these particular cases it would have been entirely without action. However, the evidence that we have before us suggests that in 17 hypochromic cases and three orthochromic cases iron was the major deficiency, and that in the three hyperchromic, 13 orthochromic and three hypochromic cases the main deficiency was the liver-Marmite factor. The original classification was made on the first count only, but, if the subsequent examinations are taken into consideration, it will be seen that it is quite justifiable on hæmatological grounds alone to modify the original classification and to transfer the three initially-orthochromic iron-responding cases to the hypochromic class and the three hypochromic Campolon-responding cases to the orthochromic class (see Graph 1). Therefore, for analysis of the other hæmatological and the ætiological data this reclassification has been made.

Summing up, therefore, we can now say of the 39 patients who improved on treatment that the main deficiency in the 20 *predominantly* hypochromic cases was iron, and in the 19 *predominantly* hyperchromic or orthochromic cases it was the liver-Marmite factor.

The deaths and failures.—The question then arises as to whether we should not take into consideration our deaths and failures. The patients who died were all but one in the orthochromic group: either they died at parturition before treatment had had time to effect improvement, or the treatment had been mixed, both iron and Campolon, and had failed, so that they cannot be placed with any certainty into either class.

Similarly with the failures, as we have already pointed out, in four of the hypochromic—all extremely hypochromic—failures only iron was given. We might take the view that their failure to react to iron disqualifies them for the iron-deficiency class, but there may be some undiscovered reason why in these cases the iron is not being absorbed and/or utilized. These extremely hypochromic cases that do not react to iron at first are not new to us; they are usually very obstinate but eventually show some improvement after a long stay in hospital. When, however, they are persistently hypochromic they do not react to liver therapy. In the fifth hypochromic case both iron and Campolon were equally ineffective. One of the orthochromic failures was a very unsatisfactory case in that there was severe sepsis: in the other, both iron and Campolon were given. Again, therefore we cannot group these cases satisfactorily.

Alternative classifications.—The characteristic blood picture of the case of anæmia that reacts to liver therapy is hyperchromic (and macrocytic), and in taking the dividing line at 32 $\gamma\gamma$ we have certainly not placed it too high, judging by ordinary standards (the normal mean MCH = 29 $\gamma\gamma$), but, as the population is not a normal one, we did consider the possibility of taking a lower dividing line. There is, however, nothing to be gained by doing this, as it would have to be lowered very considerably to bring in many more cases into the hyperchromic class, and, in addition to the three hypochromic cases, other cases with a very low MCH, e.g., case 46, responded well to liver therapy.

Again, the characteristic blood picture of iron-deficiency anæmia is hypochromic (and microcytic): we have taken the dividing line at 22 $\gamma\gamma$. This is low on the usual standards, the normal range being from about 24 to 32, but it is high on the standards of this population ($MCH = 23.4 \pm 3.1 \gamma\gamma$). If we lowered our dividing line to the 20 $\gamma\gamma$ level, we should certainly exclude all the liver-reacting hypochromic cases, but it would also exclude a number of others that were definitely iron deficient, so again there is nothing to be gained by making this adjustment.

We then considered taking one of the other hæmatological values as our criterion. We tabulated the initial mean cell volumes for the iron and the liver-Marmite groups, respectively, and found that the mean in the former was 81.1 cu. μ and in the latter 102.6 cu. μ , but there was a good deal of overlapping: in the iron group there were six cases with a higher MCV than the lowest (84 cu. μ) of the liver group and two of these were above 100 cu. μ , so that no clearer line of distinction would be obtained by taking size rather than hæmoglobin content in this series, as we had already tentatively decided.

It seemed scarcely likely that the hæmoglobin concentration would assist us much, but we tabulated this, and found the mean MCHC of the iron group to be 24.56 per cent against 27.26 per cent for the liver group. The difference is statistically significant, but here there is even more overlapping, it is obvious that this value would give little assistance in classification, and it seems probable that our choice of the MCH for this purpose was the best in the circumstances.

Conclusion.—After considering the advisability of re-adjusting our criteria for hypo- and hyperchromia and the possibility of basing our classification on other hæmatological values, we have decided to adopt, for purposes of this inquiry, a reclassification based on response to treatment, a classification which is in keeping with the general hæmatological picture and which only necessitates a slight re-arrangement from the provisional grouping of our cases.

Thus, we have divided our cases on both hæmatological and therapeutic grounds into two groups, the predominantly hypochromic that react to iron therapy (subsequently called the iron group) and the predominantly orthochromic or hyperchromic that react to liver or Marmite (subsequently called the liver-Marmite or liver group). As the deaths and failures cannot be fitted with certainty into either of these groups, we have decided to leave them out of consideration, or to classify them separately in our subsequent analyses: with this unclassified group we will include the two doubtful cases (5 and 48) and the patient who received no treatment.

ANALYSIS OF OTHER DATA.

Age incidence.—The ages given by the patients are very inaccurate, and in most cases the age recorded is an approximate estimate made by the doctor. There is apparently no distinction between the two groups.

TABLE II.

	Iron group.	Other hypo- chromics.	Liver group.	Other ortho- chromics.	TOTAL.
Under 20 years ..	4	3	2	2	11
20 but under 25 „ ..	6	2	5	6	19
25 „ „ 30 „ ..	4	..	7	3	14
30 „ „ 35 „ ..	4	1	2	1	8
Over 35 „ ..	2	..	3	..	5

Previous pregnancies.—These are shown in Table III:—

TABLE III.

	Iron group.	Other hypo- chromic cases.	Liver group.	Other ortho- chromic cases.	TOTAL.
Primigravida ..	7	3	1	2	13
2nd pregnancy ..	2	1	1	3	10
3rd „ ..	6	1	4	3	14
4th „ ..	2	1	6	3	12
5th „ ..	1	..	1	..	2
6th „ ..	1	..	1	1	3
7th or 8th pregnancy ..	1	..	2	..	3
TOTAL ..	20	6	19	12	..

We have unfortunately no figures for non-anæmic coolies with which to compare these. The table indicates that primigravidæ are liable equally with those who have had previous pregnancies to suffer from anæmia, whereas in the liver-Marmite deficient group the anæmia shows a distinct tendency to occur in the later pregnancies. This is not in keeping with our previous experience (Napier and Billimoria, *loc. cit.*), in which four out of eight hyperchromic cases were primigravidæ, but we are in the present series dealing with slightly larger figures.

Month of pregnancy.—The history regarding the month of pregnancy was very unsatisfactory and usually had to be ignored.

A rough estimate was made from the size of the uterus when the patient was first seen.

The figures, such as they are, are given in Table IV:—

TABLE IV.

Approximate month.	Iron group.	Other hypo-chromics.	Liver group.	Other ortho-chromics.	TOTAL.
3rd or 4th ..	1	1
4th „ 5th	1	..	1
5th „ 6th ..	2	..	2	..	4
6th „ 7th ..	4	3	6	2	15
7th „ 8th ..	6	1	3	6	16
8th „ 9th	1	1	3	5
Full term ..	3	..	1	1	5
Post-partum ..	4	1	5	..	10
TOTAL ..	20	6	19	12	57

There is little information to be obtained from this table except that few patients came under observation before the sixth month.

Malaria and splenic enlargement.—The district from which the patients come is a very malarious one and although there has been a considerable improvement

since anti-larval measures have been installed on a large scale, this infection is still a very important one in producing illness amongst these coolies. Actual infections were only demonstrated in two cases, but most of the patients had probably taken quinine before admission. One of these patients died during labour and the other was amongst the successfully-treated iron-deficient cases: the latter had no splenic enlargement, and neither had a positive van den Bergh reaction. We may assume that all have had malaria at some time or another, the important points being how frequently and how they have reacted to this infection.

There is little kala-azar in this district and this disease was, for all practical purposes, excluded in every case of enlarged spleen by the aldehyde test. Other causes of splenomegaly, such as leukæmia, were also excluded and the assumption is that enlarged spleen was an indication of repeated malarial infections. We believe that examination to ascertain whether the spleen is enlarged or not will give more important information than a most careful inquiry regarding recent febrile attacks, or even the examination for parasites in blood (which of course was done in every instance).

The splenic enlargement in the two groups and in 50 'normal' controls is shown in Table V:—

TABLE V.

				Iron group.	Liver group.	Total anæmics.	'Normal' controls.
Not palpable	10	3	13	35
Just palpable	5	3	8	11
Enlarged 1 inch	below costal margin	2	1	3	3
„ 2 inches	„ „ „	2	6	8	1
„ 3 „	„ „ „	4	4	..
„ 4 „	„ „ „	1	1	..
Size not recorded	1	1	2	..

Thus, there was some degree of enlargement in 24 of the 37 cases in which the size was noted and in 16 of these the spleen was an inch or more below the costal margin. However, the two series differ 'significantly' from one another, the incidence being much higher in the group that reacted to liver and Marmite. It will be seen from the figures for 'normal' controls from the same general population that the incidence of enlarged spleen in the 'iron deficient' group is not much greater than that of the general population, whereas the incidence in the 'liver-Marmite' group is distinctly and 'significantly' higher; this indicates some ætiological association between the anæmia of this type on the one hand and splenic enlargement and/or malaria on the other.

The van den Bergh.—In the absence of a colorimeter, this test was not done quantitatively, but we are accustomed to the quantitative technique, which we always use when in Calcutta, and the values given to the readings are certainly approximately correct. The 'direct' van den Bergh was negative in every case. The results of the initial 'indirect' test are shown in Table VI, together with the results in 50 'normal' controls.

TABLE VI.

Result.	Approximate concentration of bilirubin in mg. per 100 c.c.	Iron group.	Liver group.	'Normal' controls.
Negative ..	Less than 0.5 (— 1 unit)	6	4	39
(+) ..	0.5 to 1.0	9	5	6
+ ..	1.0 to 2.0	3	7	5
++ ..	2.0 to 3.0	1	2	..
+++ ..	3.0 or over	1	1	..

The van den Bergh was, thus, distinctly 'positive' in five (or 10 per cent) of the controls, in five (or 25 per cent) of the iron-deficiency cases and in 10 (or 53 per cent) of the liver-Marmite group.

The difference between the controls and the 'iron' group is not 'significant', but between the controls and the liver-Marmite group the difference is very significant. The difference between the liver-Marmite group and the 'iron' group is also just short of the significance level.

Other workers (e.g., Wills, 1931) considered that a positive van den Bergh is most probably explained by concomitant malaria, but there may be other causes for the positive van den Bergh, and on the other hand the van den Bergh test is by no means always positive when a malarial infection is present or even during a clinical malarial attack, for in neither of the two cases in this series in which malarial parasites were found was the van den Bergh test positive.

However, this combination of the association of both enlarged spleen and a positive van den Bergh test with the liver-Marmite group does seem to bring added support to the suggestion made from previous experience (Napier, 1936) that malaria plays some part in the ætiology of this hyperchromic (or orthochromic) type of anæmia.

Urobilin.—The urobilin in the urine was definitely increased in about half the cases: but there appears to be no distinction between the two groups.

Hookworm.—The stools were examined for helminthic ova. In the large majority of cases the ova of hookworm, round worm and trichuris were found. A rough estimation of the weight of infection was made by doing egg counts on a known quantity of stool.

The results are shown in Table VII:—

TABLE VII.

Eggs per gramme of stool.	Iron group.	Liver-Marmite group.	TOTAL.
<i>Nil</i> ..	2	1	3
Below 1,000 ..	4	5	9
1,000—2,000 ..	5	5	10
2,000—5,000 ..	2	3	5
5,000—or over ..	6	4	10

It is almost certain that every patient had hookworm infection, as on a single examination ova were found in 92 per cent of the stools. The important point is the weight of the infection, and, though egg counting is but a poor way of ascertaining this, it is the only method available in the circumstances. Twenty-seven per cent show an infection rate of over 5,000 ova per gramme, which we usually consider pathogenic. There is every indication as we (Napier and Das Gupta, 1937) and many other workers have shown that, where hookworm is the main factor in the production of anæmia, the degree of anæmia is correlated with the weight of the hookworm infection: in this series there is little indication of any such correlation, and, although there are six in the 'iron group' against four in the other that have heavy infections, this difference is far from 'significant'.

Gastric analyses.—These were not popular and later were given up. The usual technique was adopted, the alcohol meal being used. From six to 10 samples were taken: the results can be grouped as given in Table VIII:—

TABLE VIII.

Maximum acid free per 100 c.c.	Iron group.	Liver group.	Others.	TOTAL.
Achlorhydria— <i>nil</i> ..	1	2	..	3
Hypochlorhydria—less than 10 c.c. ..	2	3	1	6
Isochlorhydria, low—10-24 ..	3	3	5	11
„ medium—25-44 ..	6	3	1	10
„ high—45-64 ..	1	2	2	5
Hyperchlorhydria—65 or more ..	2	..	4	6
TOTAL ..	15	13	13	41

There was achlorhydria in only three cases out of the 41 tested and in these histamine was not used. In nearly two-thirds of the cases the gastric acidity was within the normal range. There was a tendency towards a lower acidity in the liver group, but the difference was far from significant statistically.

The leucocytes.—In these, there seems to be some distinction between the two groups; in the iron group 17 out of 20 have a total leucocyte count higher than the 'normal' mean of 7,110 per c.c. whereas in the liver group there are

only seven out of 19 with a count over this figure. The mean counts in the two series are $9,410 \pm 3,460$ and $7,300 \pm 3,240$ per c.mm. The difference again is 'significant'. On the differential counts in the two series there is, however, practically no difference.

The red cells.—Anisocytosis and poikilocytosis were the rule, and in the cases of the iron group were sometimes very marked. The reticulocyte count in the untreated cases averaged 2.26 per cent in the iron group and 1.52 in the liver group; the highest in either group was 5 per cent.

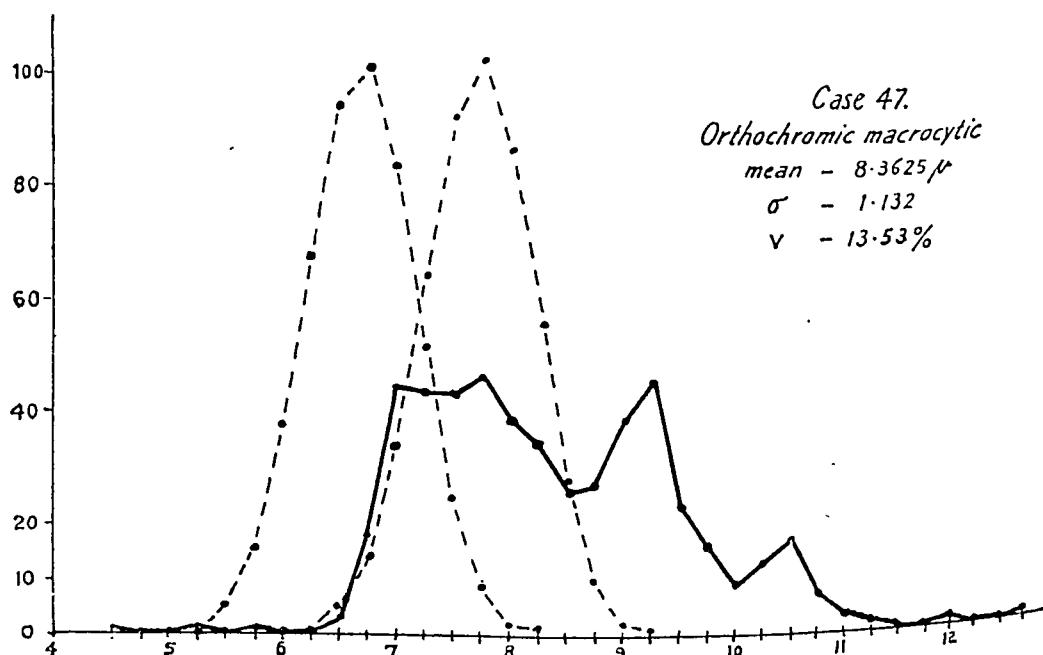
Normoblasts and occasionally erythroblasts were found in most of the cases of either group, and rarely, if ever, true megaloblasts.

Red cell diameters.—We took and kept for subsequent examination slides for all the case on admission. We had failed to find the time to examine these by Price-Jones' method, when Dr. G. Sankaran, Officiating Professor of Biochemistry, All-India Institute of Hygiene and Public Health, very kindly offered to do a number of these by the method of Hynes and Martin, which he has modified (Sankaran and Radhakrishna Rao, 1938). This method is many times more rapid than the method we have adopted hitherto and is quite reliable.

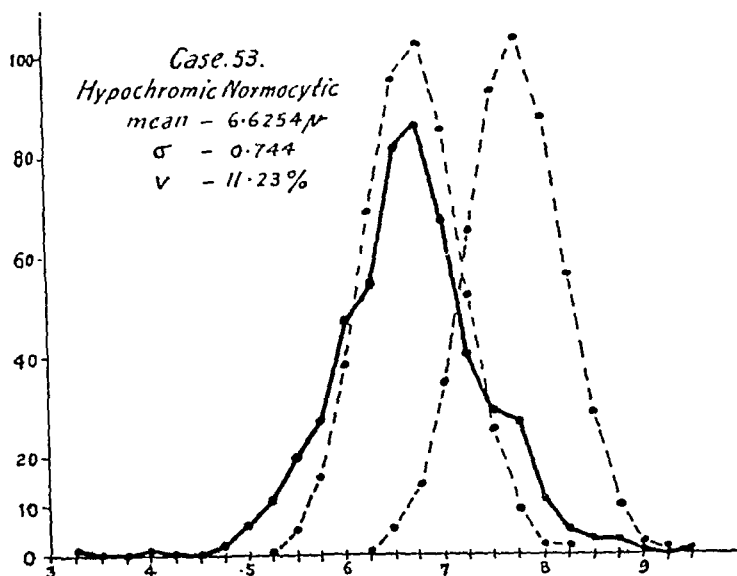
As far as we were concerned the results were a little disappointing as there seemed to be little correlation between the mean diameters and either the mean corpuscular volumes (MCV) or the mean corpuscular hæmoglobins (MCH).

Only 15 cases, three cases from each of five different groups, were done: the smallest diameter was 6.625μ (case 53) and the largest 8.363μ (case 47): the two Price-Jones' curves are shown in Graphs 36 and 37.

GRAPH 36.



GRAPH 37.



Admittedly, the mean of the three hypochromic cases was the smallest, but the mean of the three hyperchromic cases was not the largest. The means of the three cases in each of various groups are shown in the table below.

The co-efficient of variation was high in nearly every case, the highest, a hyperchromic case, being 0.1417.

TABLE IX.

Group.		Mean diameters.
Hypochromic,	iron-reacting ..	6.909 μ
Orthochromic	„ „ ..	7.026 μ
„	Marmite-reacting ..	7.668 μ
„	Campolon-reacting ..	7.481 μ
Hyperchromic	„ „ ..	7.114 μ

These observations seem to give further evidence of the very 'mixed' nature of these cases.

Sternum puncture.—Sternum puncture was done in 15 cases altogether, but circumstances prevented this being done until the investigation was well advanced and many of the patients had received treatment. In nearly all there was a relative increase in the red-cell-forming elements, more so in the iron-deficiency group than in the others.

There was no typical case of the megaloblastic hyperplasia that one sees in pernicious anæmia and sometimes in tropical macrocytic anæmia; on the other hand, erythroblasts almost equalled normoblasts in many of the cases of the Marmite-liver group.

The two types do not provide a clear-cut line of distinction: a few typical counts are given in cases in which treatment had not been commenced.

TABLE X.

Sternum puncture findings in five cases.

Serial number.	24	25	47	48	50
Total nucleated cells in thousands ..	48·0	72·5	?	48·7	?
Red cell series	44	50	45	37	32
Megaloblasts	1	0·5	3	4	7
Erythroblasts	11	5·5	19	11	12
Normoblasts	32	44	23	22	13
Granular series	40	40	44	57	57
Myeloblasts	1	2	2
Premyelocytes	0·5	0·5
Myelocytes	2	3	9	17	13
Metamyelocytes (young, band forms)	28	13·5	26	23	32

TABLE X—concl'd.

Serial number.				24	25	47	48	59
<i>Segmented</i>
Neutrophil				7.5	22	8	12	8
Eosinophil				1	1	1	2	1
Basophil	1	1
<i>Non-granular series</i>				16	10	11	6	11
Lymphocytes				11.5	7	9	5	10
Large mononuclears				3	3	2	1	1
Plasma cells				1.5

The Wassermann reaction.—This was done in 36 cases. The blood samples were sent to Major S. D. S. Greval, I.M.S., Professor of Serology at the Calcutta School of Tropical Medicine, who very kindly reported on them. The results are shown in Table XI :—

TABLE XI.

		Iron group.	Liver group.	Deaths.	Failures.	Total.
Negative	6	14	2	5	27
Doubtful	3	3
Moderately positive	4	1	5
Strongly	1	1

If the doubtful results could be classed with the positive the difference between the iron and the liver group would be significant. Though the only strongly positive case is in the liver group, there is some indication that a positive Wassermann is more frequently associated with hypochromic anæmia, but there is no indication that it was an important factor in checking progress or determining failure of treatment. Anti-syphilitic treatment was not given in any case during these investigations.

Final results of treatment in the two groups.—We have tabulated the results of the final examinations in these two groups, hæmoglobin, MCH, MCV are given in Table XII:—

TABLE XII.

	Liver group.	Iron group.
Number of cases in the group	19	20
Number of cases with more hæmoglobin than the mean of the population, i.e., 10·4 g.	7	4
Mean of final hæmoglobin readings in grammes ..	9·34	8·50
Number of cases in hypochromic class, i.e., below 22 $\gamma\gamma$	4	8
Mean of final MCHs	24·2	23·05
Number of cases in microcytic class, i.e., below 75 cu. μ	3	2
Mean of final MCVs	87·6	84·75

Many of the patients continued treatment outside and did not come again for examination, but neither this nor the time that they were under treatment is taken into account.

The more severe cases of anæmia were in the liver group but the improvement has been greater in this group. It must be remembered that nearly all the cases in this group had iron also, whereas the reverse is not true.

In the iron group, mostly hypochromic at the beginning, eight cases (40 per cent) are still hypochromic, and the mean MCH is below the average for the population, i.e., 23·42 $\gamma\gamma$; but if the liver group is also included it will be seen that the mean of all the treated cases is within 0·25 $\gamma\gamma$ of this figure. No single patient had an MCH over 30 $\gamma\gamma$ on discharge.

There was one macrocytic case amongst the iron group and none amongst the others. The MCV in the iron group now closely approximates to that of the controls, but the average size in the liver group is still above the 'normal' of this population but actually below the 'normal' for the city-dwelling Indian.

Definition of our two groups.—We are now in a position to give a more detailed definition of these two groups on the data we have presented.

Iron-deficiency group.—The patients may be of any age, between 15 and 40, and the anæmia may occur in any pregnancy, but there seems to be a slightly greater tendency amongst primigravida to suffer from this type of anæmia: it may become established at any month, but is more frequent in the latter months of pregnancy, and it may be post-partum.

There is no correlation between this anæmia and splenic enlargement: practically all patients had a hookworm infection and 30 per cent had a heavy infection, but there is little correlation between the degree of anæmia and the weight of the infection. The condition is not associated with low gastric acidity. The van den Bergh (indirect) test is usually negative.

The anæmia may be of all degrees and was even down to 2 g. per 100 c.c. of blood in some cases: the reticulocyte count in the untreated case is usually about 2 per cent. The typical blood picture is microcytic and hypochromic, and there is also a low hæmoglobin concentration in the individual cells; anisocytosis is the rule and frequently there are normoblasts: there is usually a slight but distinct leucocytosis.

There is a good response to iron therapy in the early months of pregnancy and post-partum, but in the latter months of pregnancy the response may be only slight until the uterus is empty, when rapid improvement occurs. In the ante-partum cases the improvement is accompanied by only a sub-maximal reticulocyte response or no increase in reticulocytes, but in the post-partum cases there is usually a characteristic reticulocyte response, the degree depending on the hæmoglobin level. The improvement is usually accompanied by a rise in the mean corpuscular hæmoglobin.

The Marmite-liver-deficiency group.—The patient may be of any age between 14 and 40, and the anæmia may occur in any pregnancy but it seems to be more common in the later, especially the third and fourth, pregnancies. It may be established at any month but was noted much more frequently in the third trimester, or it may be post-partum.

There appears to be a definite association between this anæmia and splenic enlargement. Practically all our patients had hookworm infection but there was no correlation between the weight of infection and the degree of anæmia. There is no association with achlorhydria, but there is some though inconclusive evidence of association with a slightly lowered gastric acidity. The van den Bergh test is usually positive.

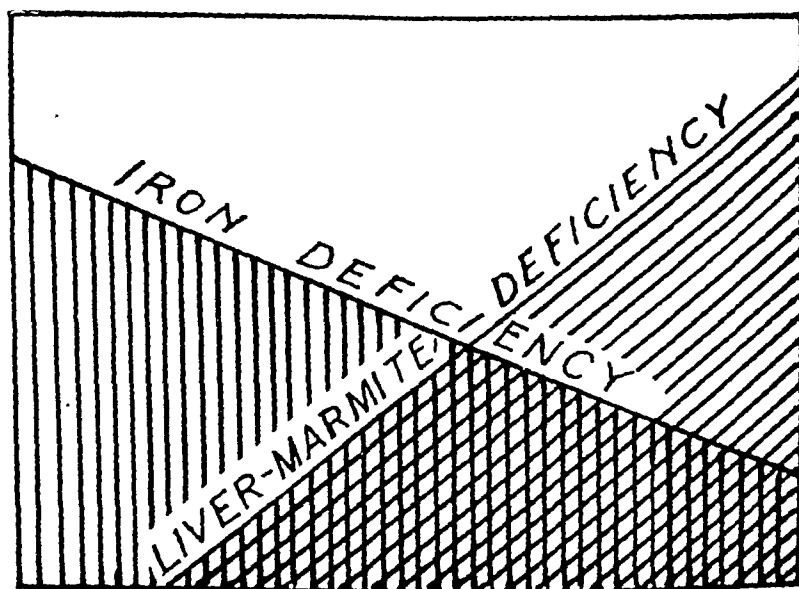
The anæmia may be of any degree and is frequently below 2 g.; it fell as low as 1.65 g. in one case. The reticulocyte count in the untreated case is low, averaging about 1.5 per cent. The typical blood picture is hyper- or orthochromic and macro- or normocytic. There is some anisocytosis and

poikilocytosis, and normoblasts and erythroblasts are seen in the peripheral blood but rarely megaloblasts. The leucocyte count is normal and there is no change in the proportions of the different cells.

There is a good response to Marmite by the mouth or Campolon by intramuscular injection, but in the later stages of pregnancy this may be counteracted or delayed until the uterus is empty: this applies particularly to Marmite. There is usually a sharp reticulocyte response, an increase in the number of red cells and a fall in the mean hæmoglobin concentration after administration of Campolon, even in the absence of any marked improvement in the hæmoglobin level.

Mixed deficiencies.—We have described above the characteristic picture of these two types of anæmia from the data that we have collected, but it is very probable that few of our cases presented a pure picture of one type of anæmia and it is quite certain that many were examples of a mixed type of anæmia in which at least two ætiological factors were at work, e.g., case 2, originally a hyperchromic anæmia improved up to a point and became hypochromic when liver was given; eventually, after iron had been given, the blood picture returned to normal.

It seems probable had there been only one ætiological factor in each case that much purer pictures than the ones we have described would be obtained; for example, the high percentage of orthochromic cases in the Marmite-liver-deficiency group is probably due to the influence of a co-existent iron deficiency. From the data we have at our disposal—admittedly defective in certain respects, for example, with reference to the effect of liver in the extremely hypochromic cases—we believe that the following diagram demonstrates the position:—



This shows iron deficiency as a constant factor, and the Marmite-liver deficiency as present in about three-quarters of the cases but dominant in just less than half.

The ætiological factors.—We need not discuss here physiological anæmia of pregnancy as it has been shown that this seldom, if ever, amounts to a deficiency of over 15 per cent of the normal amount of hæmoglobin per c.c. of blood. Dieckmann and Wegner (1934) have pointed out that the hæmoglobin in the body is increased by an average of 13 per cent during pregnancy and that the volume of plasma is increased by 20 per cent, so that there is an average deficiency of hæmoglobin per given volume of blood of only 7 per cent: they consider that at any stage of pregnancy a hæmoglobin of below 10 grammes per 100 c.c. of blood should be considered as pathological. Even allowing for the very low level of the coolie population, our least anæmic case is well below the 15-per-cent-deficiency level.

Before discussing the ætiology of the anæmias we must first consider the so-called normals. A hæmoglobin percentage of 10·40 grammes per 100 c.c. of blood cannot be considered physiological, nor can a mean corpuscular hæmoglobin of 23·42 $\gamma\gamma$. We have gone into this question at some length in previous papers (Napier and Das Gupta, 1936, 1937) and whilst coming to the conclusion that iron deficiency, determined by repeated blood loss from hookworm infection in individuals with a low iron intake, was probably an important ætiological factor, we still thought that there was some factor, possibly some other deficiency of a substance that was itself essential for normal hæmopoiesis, or one that determined the absorption and/or utilization of iron. We are continually encountering cases in our hospital experience, usually individuals of the ryot class, who have a permanently small cell: their red cell count rises to normal and often up to 6,000,000 per c.c. but their hæmoglobin still remains persistently at about the 11-gramme level.

The coolie population must be considered, therefore, to be composed of individuals suffering from an actual iron deficiency or living at the lowest level of iron balance, so that any additional demand for, or dissipation of, iron will throw over the balance against the individual and an iron-deficiency anæmia will be established.

The microcytic group.—Pregnancy will certainly supply this additional demand just as extra blood loss at parturition will the dissipation; hypochromic iron-deficiency anæmia of pregnancy is well known and the picture many of our cases present fits in very well with this condition; in view of the characteristic and rapid response to iron in these cases we need look no further for an explanation of the ætiology of their anæmia, but there were others that improved up to a point only, and few of them improved beyond the 'normal' level of the coolie population; further, the mean corpuscular hæmoglobin was in most cases only 23 $\gamma\gamma$ after considerable iron dosage, so that the effect of our hypothetical limiting factor is still apparent in these cases.

The macrocytic group.—We will now turn to the other group. The picture is very much that of Wills' macrocytic anæmia of pregnancy except that the majority are not hyperchromic macrocytic but orthochromic normocytic—this, we believe, is explained by the underlying iron deficiency—and one other important feature, namely, that in our cases the indirect van den Bergh was very frequently positive, whereas Wills gives the absence of the van den Bergh as an important distinguishing feature between the condition

she described and pernicious anæmia : in the few cases in which the indirect van den Bergh reaction was positive she attributed the fact to co-existent malaria. The important distinguishing features between our cases and pernicious anæmia are the absence of all neurological symptoms and the presence of free acid in the gastric secretions.

Though the exact nature of the deficient substance is still uncertain Wills has shown that this condition can be brought about by a deficiency of vitamin-B complex in the diet, and she has also pointed out, and we have confirmed the fact (Napier, *loc. cit.*), that a macrocytic anæmia occurs in non-pregnant women and in men which is essentially of the same nature. She, therefore, considers that the position is analogous to hypochromic anæmia of pregnancy, in that the extra demands of pregnancy determine this condition in a person who is on a diet low, but not actually deficient if the demands had been normal, in B complex.

We have shown in a previous paper (Napier and Das Gupta, 1936; Napier and Billimoria, *loc. cit.*) that, whereas the anæmia in the general coolie population was almost entirely hypochromic, amongst pregnant women there is in addition a group suffering from a hyperchromic anæmia.

In the population under investigation, which is also a tea-estate coolie population, though in a different part of Assam, a similar state of affairs almost certainly exists in the general population, and we have shown here that about half the pregnant women are suffering from a Marmite-liver-deficiency anæmia.

The occurrence of a type of anæmia amongst pregnant women that is uncommon in non-pregnant women and men suggests that pregnancy itself plays a part in its ætiology; it may simply be a matter of the extra demands of the foetus determining a deficiency in an individual on a border-line diet, that is to say, it may be a *conditioned* deficiency. On the other hand, it has been shown by Rhoads (1937) that substances which are not normally toxic will in animals kept on a deficient diet act as a toxic agent, or, in other words, there is such a thing as *conditioned* toxicity, and it is possible that this anæmia is evidence of a minor degree of toxæmia which would not affect a person protected by an efficient diet. There is the other possibility that pregnant women suffer from a temporary deficiency of the intrinsic factor in their gastric secretion, as suggested by Strauss and Castle (1933). We have no means of testing this, but, as few of our cases were achlorhydric at the time, this seems an improbable explanation.

Associated factors.—The question then arises, why do some pregnant women suffer from a simple hypochromic iron-deficiency anæmia and others from the added complications of a Marmite-liver-deficiency? There are two conditions that show a special association with the hyperchromic anæmia, namely, a positive indirect van den Bergh reaction and an enlarged spleen, and both these conditions show this association in both series, this and the one previously reported (Napier and Billimoria, *loc. cit.*). Wills (*loc. cit.*) is emphatic that a positive van den Bergh does not occur in her anæmia, and that when it is present it is evidence of complicating malaria. If we accept the view that in the pure Wills' Marmite-factor-deficiency anæmia, the van den Bergh is negative, then we must assume that the positive van den Bergh is due to some commonly associated condition; the further association with

enlarged spleen makes it difficult to avoid the conclusion that this condition is malaria. As this double association is very definitely less where the hypochromic iron-deficiency anæmia is concerned, we have a strong suggestion that chronic malaria is an important ætiological factor in the hyperchromic anæmia.

We do not propose here to enter into a discussion on the pathology of chronic malaria and why certain changes occur in some individuals and yet not in others who as frequently suffer acute attacks, but our suggestion is that it is not the acute attack—which is often not accompanied by a positive van den Bergh reaction—but the chronic state with the damaged reticulo-endothelial tissue in the spleen and elsewhere that is most likely to be the determining factor in producing this anæmia.

There is the evidence in the positive van den Bergh reaction of excessive blood destruction. Is it possible that during this process some substance, which is not synthesized in the body but which is essential to normal hæmopoiesis, is destroyed to such an extent that in the presence of a poor diet it becomes deficient?

On the other hand, it has often been observed that people of certain races rapidly acquire an immunity to the effects of malaria though parasites can always be found in large numbers in their peripheral blood, whereas others develop enlarged spleen and rapidly become the subjects of chronic malarial cachexia. Is it possible that the dietary factor is the important one, and that those on an adequate diet are protected against the ill effects of malaria, whereas the others are not, and are in a state of *conditioned* toxicity?

The dietary factor.—In our previous investigations we attempted to get information about their diet from the patients themselves, but we found that this was far too vague to be of any value. We, therefore, approached Professor Ellis C. Wilson of the All-India Institute of Hygiene and Public Health and it was arranged that one of the diet survey officers who had been placed to work under him should be sent to Dewan to carry out a dietary survey amongst the population from which our cases came. The result of this survey, which was carried out by Dr. Mitra, can be summarized as follows:—

The total calories were only a little below the standard usually considered necessary for Indian workers, but were mainly derived from carbohydrates: the total protein intake was low and the animal protein almost a negligible figure. Total fat was also very low, below 12 grammes, and most of it was of vegetable origin.

Of the minerals calcium was exceptionally low, being only about one-sixth of the usual requirements; the figure for iron was low, though adequate if it was all in available form, but there was some doubt on this point. Of the vitamins, the intakes of A and B complex were low, though apparently not actually deficient judged on most standards, but the vitamin-C intake was very low and, as this was mainly derived from vegetables that were cooked, Professor Wilson seemed to think that saturation with this vitamin was improbable.

Physical examination did not show any very striking evidence of deficiency though xerophthalmia was fairly common, but by means of the A. C. H. index about 30 per cent of the children were 'selected'.

Thus, we have evidence that the population as a whole were living on a diet relatively deficient in a number of essentials. The most striking deficiency was in calcium and our attention has been attracted to this because certain work recently carried out has shown that calcium aids the absorption and/or utilization of iron (Orten, Smith and Mendel, 1935). A second point was the possible vitamin-C deficiency. Either one of, or both, these deficiencies may, we feel, possibly explain the under-hæmoglobinized blood cell of this population, but in view of the fact that there are ways of testing blood calcium and vitamin-C saturation, it would be absurd to base any conclusion on this indirect evidence, and when the opportunity arises we shall certainly explore these possibilities.

With regard to the special hyperchromic anæmia we have already suggested that this may be due to a *conditioned* deficiency and there is evidence that none of these people take an excess of vitamin-B complex, or for that matter of any of the important food factors.

To conclude this section on diet, we can say that there is evidence which indicates that this may be a very promising line of investigation.

CONCLUSION.

The population of pregnant tea-garden coolie women as a whole has a low level of hæmoglobin associated with a small pale red blood cell: conditioned iron deficiency, brought about by continuous blood loss from hookworm infection in the presence of a low iron intake, will account for this in part, but not, we believe, altogether, and we consider it possible that a low calcium and/or a low vitamin-C intake may be associated factors.

The anæmia amongst pregnant women can be divided into two groups, characteristically microcytic hypochromic and macrocytic hyperchromic, respectively; the former is an exaggeration of the anæmia in the normal population brought about by the extra demands of pregnancy, but the factors concerned in the latter are not quite so evident, though the response to treatment suggests—and a dietary survey does not negative the suggestion—that there is a relative deficiency in important food factors probably associated with the vitamin-B complex, and other observations indicate that this deficiency is conditioned by the presence of the foetus with its extra demands for substances essential for hæmopoiesis (or less probably its toxic influence) and/or by a state of chronic malaria.

Whilst characteristic examples of each type of anæmia are seen, they seldom occur in 'pure' state and in the vast majority of cases deficiencies of both groups are evident and require rectifying.

ACKNOWLEDGMENTS.

We must first of all thank Dr. G. Fraser for his whole-hearted co-operation. It would have been quite impossible to have undertaken this inquiry without his assistance and without the excellent organization of his central hospital.

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Our thanks are due to Dr. Sankaran for his co-operation in the matter of blood-cell measurements and to his assistant, Dr. M. N. Rao, who actually made the measurements.

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Serial number.	Hæmoglobin in grammes per 100 c.c. of blood.	Red cells in millions per cu. mm.	Reticulocytes in per cent of red cells.	Mean corpuscular volume in cubic micra.	Mean corpuscular hæmoglobin in micromicrograms.	Mean corpuscular hæmoglobin concentration in per cent.	White cells (plus nucleated red cells) in thousands per cu. mm.	Polymorphonuclears in per cent of nucleated cells.	Lymphocytes in per cent of nucleated cells.	Erythroblasts in per cent of nucleated cells.	Normoblasts in per cent of nucleated cells.	Other abnormalities in blood picture.
1	5.2	1.77	0.7	86.2	29.5	34.2	4.7	35	37	12	8	..
2	2.75	0.77	4.6	113.2	35.7	31.5	4.8	41	35	8	11	..
3	2.9	1.32	13.6	107.3	21.1	20.5	8.2	63	22	4	7	..
4	2.75	0.94	5.0	98.6	30.6	29.7	13.1	53	25	9	8	..
5	4.3	1.33	0.6	114.7	31.3	27.9	7.6	64	17	3	9	..
6	3.85	1.46	4.8	97.1	26.4	27.2	12.2	46	43	3	3	..
7	3.85	1.44	2.4	102.2	26.7	26.2	5.4	39	26	21	9	..
8	6.3	2.12	0.4	105.4	29.8	28.3	5.7	42	30	—	—	E 26
9	7.6	3.50	1.0	84.1	21.8	26.0	7.5	56	25	—	—	E 16
10	6.3	3.28	2.0	83.1	19.3	23.2	15.4	81	13	—	—	..
11	4.7	1.48	1.4	103.1	31.6	30.6	8.6	31	63	2	—	..
12	6.2	3.21	4.4	74.7	19.3	25.8	7.5	47	31	2	—	E 16
13	6.6	3.37	1.6	67.9	19.6	28.8	4.4	69	16	—	—	M. T.
15	5.4	2.28	3.0	83.7	23.5	28.1	3.0	45	37	—	2	E 14
16	4.3	1.59	0.4	82.3	26.8	32.3	8.1	56	30	3	1	..
17	1.9	0.55	3.0	158.5	35.1	22.1	7.6	67	22	—	—	My
18	3.3	1.31	1.4	87.4	25.1	28.8	10.5	60	24	9	1	M. T.
19	3.0	1.17	2.2	102.6	25.9	25.2	3.4	61	27	5	2	..
20	3.7	1.70	1.4	83.4	21.8	26.2	7.7	59	36	—	—	..

A = Hypochromic.
 B = Orthochromic.
 C = Hyperchromic.

E = Eosinophiles in per cent of nucleated cell.
 M. T. = Malignant tertian rings in peripheral blood.
 My = Myelocytes in peripheral blood.
 Ach = Achlorhydia.
 H (—) = Hypochlorhydia.
 N (—) = Isochlorhydia (low).
 N = " (medium).
 N (+) = " (high).
 H (+) = Hyperchlorhydia.

COL.

Van den Bergh reaction (indirect).	Urobilin in urine.	Gastric analysis.	Hookworm ova in thousands per gramme of stool.	Wassermann reaction.	Age.	Gravida.	Month of pregnancy.	Splenic enlargement in inches.	Oedema.	Albumin in urine.	Fever.	Complications.	Group.	REMARK.
++	+	Ach	0.6	-	25	4th	P. P.	2	-	-	+	-	B	..
+++	+	H (-)	8.4	-	28	4th	P. P.	P	-	-	-	-	C	..
+++	++	N (-)	0.8	..	30	3rd	P. P.	2	-	-	+	-	A	..
+	-	20	1st	6-7	1	++	-	+	+	B	D
+	++	N (+)	15.2	..	26	6th	8-9	2	++	-	-	-	B	..
(+)	++	N	34.4	+	19	1st	F. T.	1	++	T	+	++	B*	..
(+)	-	Ach	26.8	-	26	4th	6-7	-	+	-	+	+	B	..
(+)	(+)	N (+)	2.6	..	35	8th	5-6	2	++	-	+	++	B	..
-	-	N	9.4	..	23	3rd	8-9	-	-	-	-	+	A	..
(+)	++	H (+)	14.6	..	22	3rd	P. P.	P	+	-	-	-	A	..
-	(+)	N (-)	10.8	..	22	2nd	7-8	-	++	-	-	-	B	..
-	(+)	H (-)	-	..	19	1st	6-7	2	-	-	-	-	A	F
-	(+)	Ach	17.6	-	18	1st	6-7	-	-	-	+	-	A	..
(+)	++	N (-)	0.6	-	25	2nd	7-8	-	-	-	-	-	B	F
(+)	(+)	N	10.6	..	29	3rd	P. P.	P	-	-	+	-	B*	..
(+)	(+)	H (-)	4.0	-	25	6th	P. P.	3	-	-	+	-	C	..
(+)	+	N (+)	0.6	..	22	2nd	6-7	P	+	-	-	+	B	D
+	+	N	8.4	-	20	2nd	6-7	3	-	-	-	-	B	..
++	++	N	1.6	-	30	4th	F. T.	4	++	-	-	-	A*	..

P = Palpable.

T = Trace.

P. P. = Post-partum.

F. T. = Full term.

D = Died.

F = Failed to respond to treatment.

* Indicates reclassified according to response to treatment.

Serial number.	Hæmoglobin in grammes per 100 c.c. of blood.	Red cells in millions per cu. mm.	Reticulocytes in per cent of red cells.	Mean corpuscular volume in cubic micra.	Mean corpuscular hæmoglobin in micromicro-grams.	Mean corpuscular hæmoglobin concentration in per cent.	White cells (plus nucleated red cells) in thousands per cu. mm.	Polymorphonuclears in per cent of nucleated cells.	Lymphocytes in per cent of nucleated cells.	Erythroblasts in per cent of nucleated cells.	Normoblasts in per cent of nucleated cells.	Other abnormalities in blood picture.
21	3.85	1.69	0.4	80.7	22.8	20.9	5.9	58	27	1	4	..
22	6.9	3.63	3.6	81.1	18.9	23.3	10.3	69	28	—	—	..
23	6.6	2.89	1.2	84.9	22.8	27.3	6.7	48	25	—	24	..
24	6.3	3.84	0.4	61.0	16.5	27.0	9.4	40	49	1	—	..
25	6.2	3.55	2.2	73.7	17.4	23.6	7.9	63	30	—	—	..
26	3.6	2.46	4.2	71.0	14.5	20.5	12.6	37	38	1	1	E 19
27	6.6	3.21	3.0	79.8	20.6	25.8	9.5	50	40	1	—	..
28	3.85	1.32	0.8	107.3	29.2	27.2	7.4	65	26	1	—	..
29	2.2	0.95	1.6	97.6	23.2	23.7	3.7	40	45	—	12	..
30	4.5	1.55	0.2	105.5	29.2	27.7	6.8	61	35	—	—	..
31	5.9	1.86	0.6	102.6	31.7	30.9	10.4	77	21	—	—	..
32	7.0	3.3	1.8	76.0	21.3	28.0	6.5	66	21	2	—	..
33	4.1	2.16	1.0	88.3	19.1	21.6	10.8	64	33	—	—	..
34	4.7	2.89	2.6	77.3	16.2	20.9	9.4	79	16	1	—	..
35	3.9	2.04	1.6	86.0	18.9	22.1	8.1	68	22	3	3	..
36	2.6	1.23	0.4	84.2	21.2	25.2	5.3	57	38	3	1	..
37	5.5	3.53	0.2	62.0	15.6	25.2	8.1	63	25	—	2	..
38	6.7	3.16	2.0	82.8	21.3	25.8	7.8	59	35	2	1	..
39	6.2	2.38	2.4	103.1	26.0	25.2	4.7	69	26	—	—	..
40	6.1	2.5	1.2	96.0	24.2	25.2	14.1	69	21	2	1	..

A = Hypochromic.
B = Orthochromic.

E = Eosinophiles in per cent of nucleated cell.
H (—) = Hypochlorhydria.
N (—) = Isochlorhydria (low).
N = " (medium).
N (+) = " (high).
H (+) = Hyperchlorhydria.

COL—contd.

Van den Bergh reaction (indirect).	Urobilin in urine.	Gastric analysis.	Hookworm ova in thousands per gramme of stool.	Wassermann reaction.	Age.	Gravida.	Month of pregnancy.	Splenic enlargement in inches.	Edema.	Albumin in urine.	Fever.	Complications.	Group.	REMARK.
(+)	—	N (—)	29	3rd	7-8	1	—	—	+	+	B	D
(+)	—	N (—)	1.0	+	34	4th	7-8	1	—	—	—	—	A	..
—	—	..	1.8	—	37	3rd	6-7	P	+	+	—	+	B	..
+	—	H (—)	28	4th	6-7	—	—	—	—	—	A	..
—	—	N	1.6	..	35	3rd	7-8	—	—	—	—	—	A	..
++	(+)	H (+)	5.2	+	16	1st	3-4	—	—	—	—	—	A	..
(+)	—	H (+)	1.0	+	30	4th	8-9	1	—	—	—	—	A	F
—	—	N	22.2	..	14	1st	7-8	P	++	—	+	—	B	D
++	++	N (—)	2.4	—	22	3rd	P. P.	—	—	—	—	—	B	D
+	+	H (—)	0.2	—	32	5th	6-7	2	+	—	—	—	B	..
—	—	N (—)	0.4	—	26	4th	7-8	..	—	—	+	—	B	..
—	—	H (+)	2.4	—	18	1st	6-7	—	—	—	—	—	A	F
++	+	N (—)	0.5	—	22	3rd	P. P.	—	—	—	+	++	A	F
—	(+)	H (+)	1.6	—	24	2nd	7-8	—	—	—	—	—	A	F
—	—	N	—	—	20	1st	7-8	—	—	—	+	+	A	..
+	+	N	0.4	..	18	2nd	4-5	2	+	—	+	—	A*	..
+	+	N	—	(+)	28	6th	7-8	P	—	—	—	—	A	..
(+)	+	N (+)	1.4	—	22	1st	6-7	—	—	—	—	—	A	..
(+)	+	N (—)	6.0	—	22	3rd	7-8	3	—	+	+	++	B	F
+	—	N (+)	2.0	..	23	3rd	7-8	3	—	—	—	—	B	..

P = Palpable.

P. P. = Post-partum.

D = Died.

F = Failed to respond to treatment.

* Indicates reclassified according to response to treatment.

Serial number.	Hæmoglobin in grammes per 100 c.c. of blood.	Red cells in millions per cu. mm.	Reticulocytes in per cent of red cells.	Mean corpuscular volume in cubic micra.	Mean corpuscular hæmoglobin in micromicrograms.	Mean corpuscular hæmoglobin concentration in per cent.	White cells (plus nucleated red cells) in thousands per cu. mm.	Polymorphonuclears in per cent of nucleated cells.	Lymphocytes in per cent of nucleated cells.	Erythroblasts in per cent of nucleated cells.	Normoblasts in per cent of nucleated cells.	Other abnormalities in blood picture.
41	5.9	2.9	5.0	76.5	20.5	26.8	7.6	60	30	—	1	..
42	6.6	2.7	1.2	84.8	24.4	28.8	6.1	42	45	1	3	..
43	4.5	2.2	4.4	89.0	21.1	23.8	11.9	55	34	—	5	..
44	3.0	1.5	0.0	90.2	20.9	23.1	9.8	53	28	2	2	..
45	4.8	2.06	0.2	84.7	23.3	28.2	5.7	70	16	—	1	..
46	3.0	1.18	0.4	101.7	25.6	25.2	6.2	67	32	—	—	..
47	2.8	1.02	4.6	106.9	27.0	25.2	15.8	53	28	1	4	..
48	5.2	1.81	1.2	93.4	28.9	30.9	5.9	—	—	..
49	3.0	1.46	1.2	82.2	20.7	25.2	5.5	63	31	—	1	..
50	5.1	2.02	0.0	91.7	25.3	27.5	4.3	72	23	—	—	..
51	5.9	2.30	0.2	94.0	25.5	27.1	9.4
52	2.1	0.95	0.4	104.0	21.7	20.8	9.9	45	40	3	7	..
53	4.5	2.30	2.4	75.2	19.5	26.0	7.2	42	46	—	—	..
54	6.2	3.36	1.0	69.8	18.4	26.4	6.9	68	24	—	—	..
55	2.3	0.6	4.6	145.3	39.0	26.8	5.8	63	23	2	10	..
56	3.85	2.03	4.8	80.5	19.0	23.6	19.3	61	31	1	3	..
57	5.8	2.13	0.0	94.7	27.1	28.7	6.2	59	38	—	—	..
58	5.6	1.89	1.2	95.2	29.8	31.3	4.1

A = Hypochromic.
 B = Orthochromic.
 C = Hyperchromic.

N (—) = Isochlorhydria (low).
 H (—) = Hypochlorhydria.

COL—concd.

Van den Bergh reaction (indirect).	Urobilin in urine.	Gastric analysis.	No. of worms in thousands per gramme of stool.	Wassermann reaction.	Age.	Gravida.	Month of pregnancy.	Splenic enlargement in inches.	Edema.	Albumin in urine.	Fever.	Complications.	Group.	REMARK.
—	—	H (—)	0.2	—	24	1st	7-8	1	—	—	—	—	A	..
(+)	—	N (—)	1.0	—	18	1st	6-7	2	—	—	—	—	B	..
—	—	N (—)	2.0	(—)	46	8th	P. P.	..	—	—	—	—	A	..
—	—	..	—	—	22	4th	P. P.	—	—	—	+	++	A*	..
(—)	(+)	..	1.0	—	34	5th	6-7	2	—	—	—	—	B*	..
—	—	..	4.6	—	27	2nd	P. P.	2	—	—	+	—	B	..
—	—	..	0.2	—	38	7th	5-6	3	—	—	—	—	B	..
(+)	(+)	..	1.6	..	19	4th	8-9	—	++	—	—	—	B	..
—	—	—	16	1st	P. P.	2	+	—	—	++	A	D
+	—	++	28	3rd	8-9	1	—	—	—	—	B	..
—	24	2nd	F. T.	..	++	—	—	+	B	D
(+)	+	..	4.0	..	30	3rd	7-8	P	—	—	+	++	A	..
(—)	0	..	0.6	—	18	1st	P. P.	—	—	—	—	+	A	..
(+)	+	..	1.0	(+)	20	2nd	5-6	—	—	—	—	—	A	..
—	++	..	1.4	—	22	3rd	P. P.	P	++	—	—	+	C	..
+	(+)	..	2.0	—	25	2nd	P. P.	—	—	—	+	+	A	..
+	(+)	..	0.2	—	37	4th	8-9	—	+	—	—	—	B	D
—	23	4th	7-8	..	—	—	—	—	B	..

P = Palpable.
P. P. = Post-partum.
F. T. = Full term.
D = Died.

* Indicates reclassified according to response to treatment.

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INVESTIGATIONS ON THE VARIATION OF VIBRIOS IN THE HOUSE FLY.

BY

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[Received for publication, September 23, 1938.]

IN the course of recent investigations on vibrios and particularly on *V. cholerae* many workers have failed to find any evidence of serological or other variations except the change to 'rough' type of determined serological nature. On the other hand, other workers have from time to time brought forward evidence in support of occurrence of serological and other variations in these organisms.

From the epidemiological point of view this matter is of considerable importance. If *V. cholerae* is subject to material variations, the maintenance of the virus in an endemic region or in an inter-epidemic period, in a form not readily recognizable, would be possible. If not, in the study of the epidemiology of cholera, attention can be focused entirely on typical *V. cholerae*, eliminating atypical vibrios from consideration.

Most of the work on the subject had been carried out before the fact had been recognized that the separate determination of 'O' agglutination was essential in the serological examination of the vibrios and that an 'H' fraction in common with that of *V. cholerae* was present in many vibrios which can be isolated from water and other sources under conditions in which cholera contamination can be excluded.

It has long been recognized that the house fly may play a part in the dissemination of cholera as a mechanical carrier of the vibrio. Since 1885, when Maddox first made a scientific study of this subject, much evidence on the point has been presented. Generally speaking, the observations made have tended to show that house flies can, at most, play a limited rôle in transmitting infection from place to place. On the other hand, Gill and Lal (1931) as a result of

their combined field and laboratory investigations have suggested that flies might play a more fundamental part in the epidemiology of cholera by acting as biological carriers of the organism.

The present studies were undertaken with a view to investigating what changes, if any, could be detected in the vibrios after passage through the flies by employing more modern techniques.

Breeding and sterilization of flies.—The flies were caught and bred in the laboratory by the technique described by Gill and Lal (*loc. cit.*). Much more stringent measures were, however, taken to raise flies free, as far as possible, from bacterial and bacteriophage contamination so that these extraneous infections might not interfere with subsequent investigations. For the sterilization of the eggs beginning was made with disinfectants of the coal-tar group, formalin, and chlorine solution but indifferent results were obtained. Best results were achieved with Murdoch and Smart's (1931) technique. The mass of eggs was first broken up by shaking it with modified Dakin's solution containing 0.39 per cent chlorine and 1.6 per cent anhydrous sodium carbonate. The eggs, so separated, were treated in Murdoch and Smart's apparatus for half an hour with hydrarg. perchlor. solution (1 in 1,000) to which $\frac{1}{2}$ per cent hydrochloric acid had been added. They were then thoroughly washed, and allowed to hatch out under aseptic condition in a medium of mashed vegetable placed in a bottle through which sterile air was passed. The air was made sterile by drawing it successively through a cylinder containing sulphuric acid and L₃ candle. When the larvæ pupated in the bottles, they were thoroughly washed in 5 per cent carbolic acid and placed in a tall cage in which adult insects were able to fly about. They were fed on sterilized milk. Several generations were bred in this way. Considerable difficulty was encountered in effecting sterilization with regard to spore-bearing aerobes and cocci and the results were not completely satisfactory in this respect but it was found possible to eliminate altogether Gram-negative organisms from the eggs although in adult flies such organisms could occasionally be detected. However, the insects were invariably found to be free from contamination with bacteriophage or with organisms even remotely resembling *V. cholerae*. The flies used in the experiments belonged to genus *Musca*.

The experimental procedure was as follows:—

Batches of laboratory-bred flies containing from 15 to 25 insects were fed on phage-free vibrios of known characteristics by Gill and Lal's technique. At intervals of 6, 12, 24, and 48 hours (in some experiments daily up to six days) a few insects were singed, crushed, and plated in nutrient medium. A number of colonies were picked up. The organisms thus isolated were carefully studied to find out whether there was any change in their characteristics. They were also examined for possible contamination with bacteriophage during the experiment. The following characteristics of the vibrios were studied:—

Colonial appearance.

Morphology.

Sugar reactions using saccharose, mannose, and arabinose.

Serological reaction against Inaba and homologous 'O' and 'H' & 'O' sera.

Metabolism, Linton *et al.*'s (1936) technique and classification were followed.

Chemical structure.

The tests for metabolic activity and the examinations of protein and polysaccharide structure were carried out by the associated Inquiry under Dr. R. W. Linton. The chemical classification given is that of Linton *et al.* (1935) based on the determination of two types of protein and three types of polysaccharides.

Four series of experiments were conducted with the following strains:—

- | | |
|--|--|
| A. <i>Vibrio</i> isolated from a case of cholera. It agglutinated with Inaba 'O' and 'H' & 'O' sera. | |
| B. <i>Vibrio</i> isolated from a tank in Diamond Harbour area. | } All these strains were inagglutinable with Inaba 'O' and 'H' & 'O' sera. |
| C. <i>Vibrio</i> from a healthy carrier. | |
| D. <i>Vibrio</i> isolated from a tank in Calcutta. | |

A. EXPERIMENTS WITH A NON-AGGLUTINATING *VIBRIO* ISOLATED FROM A CASE OF CHOLERA.

Experiments in this series were conducted with the strain which had been isolated from a cholera case (No. 610 S. T. M. obtained through the courtesy of Major C. L. Pasricha, I.M.S.).

Originally it had the following characteristics:—

Colonial appearance: Smooth, translucent, entire edge.

Morphology: Comma-shaped vibrio.

Sugar reaction: Saccharose +, mannose +, arabinose — (Heiberg group I).

Serological reaction: Agglutinated to full titre with Inaba 'O' and 'H' & 'O' sera.

Metabolism: Respiration 12.2 (cm. of oxygen consumed per mg. of bacteria), glycolysis 7.3 (cm. of CO₂ given off per mg. of bacteria) (Linton's group I).

Chemical structure: Polysaccharide containing galactose and protein I (Linton's chemical group I). 'Phage free.

Five experiments were conducted with this strain. *Vibrios* could not be recovered from the flies more than 48 hours after feeding except in one instance when it was isolated after five days. The variations observed are described below:—

Colonial appearance.—In two experiments colonies of organisms recovered 24 and 48 hours after feeding exhibited slight opacity, in others no difference was noticed.

Morphology.—On four occasions the organisms recovered 24 and 48 hours after feeding were found to be shorter, thicker and less curved, when isolated, but they tended to revert to the original form on sub-culture.

Sugar reaction.—No change was observed in any of the strains isolated.

Serological reaction.—No change was observed when tested against homologous and Inaba 'O' and 'H' & 'O' sera, titre remaining the same.

TABLE I.

Metabolic activity and chemical structure of certain strains of vibrios recovered after passage of case strain No. 610 through flies.

Passage number.	Strain number.	Period of isolation after feeding (hours).	METABOLISM.		Linton's metabolic group.	Chemical structure.	Linton's chemical group.
			Respiration.	Glycolysis.			
Original	610	..	12.2	7.3	I	Galactose, protein I.	I.
1	2	Immediately	12.5	7.5	I
	3	6	8.5	5.4	VI
	4	6	10.0	7.1	VI
	5	6	7.6	5.4	II
2	6	12	13.8	1.2	V	Galactose, protein I.	I.
	7	24	10.4	1.6	V
	8	48	12.0	3.0	V	Glucose and galactose, protein I.	Mixed I and VI.
3	9	6	9.5	7.3	VI
	10	12	12.5	4.5	V
	11	24	11.0	5.0	V or VI
	13	120	7.5	5.5	II	Glucose, protein II.	V.

Metabolism.—The results of metabolic tests are shown in Table I. It will be observed that definite variations have occurred. Generally speaking, there was a tendency towards decrease of glycolytic power.

Chemical structure.—The chemical examination of strains Nos. 6, 8, and 13 was carried out, *vide* Table I. It will be seen that strain No. 13 isolated five days after feeding (first passage) changed to Linton's chemical group V which involve changes in the polysaccharide as well as in the protein.

None of them showed any evidence of contamination with bacteriophage.

B. EXPERIMENTS WITH A NON-AGGLUTINATING VIBRIO ISOLATED FROM A TANK.

Experiments were conducted with strain No. 2252 isolated from a tank in one of the experimental villages in Diamond Harbour area. The tank was habitually used for drinking purposes by 429 persons. There was no case of

cholera in the village at the time of isolation. The main characteristics of this organism were:—

Colonial appearance: Semi-opaque, smooth, entire edge.

Morphology: Comma-shaped vibrio.

Sugar reaction: Saccharose +, mannose +, arabinose — (Heiberg's group I).

Serological reaction: Non-agglutinating against Inaba 'O' and 'H' & 'O' sera.

Metabolism: Respiration 13.2, glycolysis 0.0 (Linton's metabolic group IV).

Chemical structure: Polysaccharide containing glucose and protein II (Linton's chemical group V). Phage free.

Ten serial passages of 48 hours each were made through the flies except in two instances when the duration of passages was shorter. The following results were obtained:—

Colonial appearance.—Transparent colonies were observed twice with the recovered organisms. These did not show any serological or biochemical variations, and tended to revert to the original type on sub-culture.

TABLE II.

Metabolic activity and chemical structure of certain strains of vibrios recovered after passage of tank strain No. 2252 through flies.

Passage number.	Strain number.	Period of isolation after feeding (hours).	METABOLISM.		Linton's metabolic group.	Chemical structure.	Linton's chemical group.
			Respiration.	Glycolysis.			
Original.	2252	..	13.2	0.0	IV	Glucose, protein II.	V.
1	30	6	10.0	1.0	V
	31	12	13.0	0.0	IV
	32	24	11.0	1.0	V
	33	48	11.0	1.8	V
2	34	6	8.0	2.0	III
	35	12	10.0	2.0	V
	36	24	10.0	1.0	V
	37	48	11.0	2.0	V

TABLE II—*contd.*

Passage number.	Strain number.	Period of isolation after feeding (hours).	METABOLISM.		Linton's metabolic group.	Chemical structure.	Linton's chemical group.
			Respira- tion.	Glyco- lysis.			
3	38	6	8.0	3.0	III
	39	12	10.0	1.0	V
	40	24	10.0	3.0	V
	41	48	9.8	3.0	V
4	42	6	14.2	9.4	I	Galactose, protein I.	I.
	43	12	10.0	1.9	V
	44	24	11.4	0.0	IV
	45	48	12.0	2.0	V
5	46	6	12.1	2.0	V
	47	24	10.2	0.2	IV
	48	48	12.1	1.5	V
6	49	24	13.0	1.9	V
	50	48	11.7	0.8	IV
7	42A	48	10.3	1.1	V
	42B	48	11.1	2.1	V	Galactose, protein II.	IV.
	42C	48	9.1	3.1	V
	42D	48	12.2	0.3	IV
8	42A1	24	12.6	5.9	VI
	42B1	24	11.7	7.9	I or VI
	42C1	24	11.4	6.0	VI
	42D1	48	12.4	9.0	I
	42E1	48	10.6	3.9	V
	42F1	48	13.4	0.7	IV
	42G1	48	12.7	2.4	V

TABLE II—*concl'd.*

Passage number.	Strain number.	Period of isolation after feeding (hours).	METABOLISM.		Linton's metabolic group.	Chemical structure.	Linton's chemical group.
			Respiration.	Glycolysis.			
9	42A2	48	10.3	3.9	V
	42B2	48	8.5	1.4	V
	42C2	48	9.4	3.1	V
	42D2	48	11.7	2.3	V
	42E2	48	12.0	3.5	V
10	42C3	24	9.3	2.5	V
	42D3	24	9.1	5.2	VI
	42E3	24	8.2	4.1	III
	42F3	48	7.5	3.6	III
	42G	48	7.7	4.5	II
	42H	48	8.9	5.2	VI	Galactose, protein II.	IV.

Morphology.—The same type of variations as observed in the previous experiment was also noticed in this series on five occasions when organisms recovered after 24 and 48 hours were examined.

Sugar reaction.—No change was observed in the whole series.

Serological reaction.—No change was observed against the homologous 'O' and 'H' & 'O' sera, agglutination titre remaining unaltered.

Metabolism.—The result of the metabolic tests is given in Table II. The results obtained were very interesting. In the fourth passage the metabolic activity of the strain isolated six hours after feeding suddenly increased. This strain was used for the next passage but the recovered strains showed reversion to low metabolic figure. Strains isolated during the 6th and the 7th passages also exhibited low metabolism, but some of the organisms recovered after the 8th passage again gave high figures. Most of the strains recovered after the 10th passage again gave higher metabolic figures, though none behaved like Linton's group I. Strains Nos. 42, 42B1, and 42D1, which showed high metabolism, were tested a number of times when kept under laboratory conditions. They showed reversion to the original figure.

Chemical structure.—Chemical examination of the following strains was carried out (*vide* Table II).

Strain No. 42	recovered	after the 4th	passage.
„ „ 42B	„	„ „ 7th	„
„ „ 42H	„	„ „ 10th	„

It will be seen that strain No. 42 which originally belonged to Linton's chemical group V had, after the 4th passage through the fly, undergone a complete change both with respect to its polysaccharide and protein. It could now be classed as group I. This change, however, was not permanent as strains Nos. 42B and 42H which were derived from it after the 7th and the 10th passages, respectively, were found to contain protein II though polysaccharide remained unaltered. These organisms thus belonged to Linton's chemical group IV.

The recovered strains were also found to be 'phage free.

C. EXPERIMENTS WITH A NON-AGGLUTINATING VIBRIO OBTAINED FROM A HEALTHY CARRIER.

Strain No. 86 was used for this experiment. It was isolated from a healthy carrier during routine examination of stool in Diamond Harbour area. There was no cholera there at that time.

The strain had the following characteristics:—

Colonial appearance: Semi-opaque, smooth, entire edge.

Morphology: curved rod.

Sugar reaction: Saccharose +, mannose —, arabinose — (Heiberg's group II).

Serological reaction: Inagglutinable against Inaba 'O' and 'H' & 'O' sera.

Metabolism: Respiration 5.1, glycolysis 2.6 (Linton's metabolic group III).

Chemical structure: Polysaccharide containing galactose, glucose, and protein II (Linton's chemical group mixed IV and V). 'Phage free.

The experimental procedure was slightly changed in this case. Unlike the previous experiments isolations were made only after 24 hours of feeding and thus the duration of the passage was reduced to 24 hours. After each passage many colonies were picked up. Each time the strain showing highest glycolytic figure was selected for the net passage. Altogether six passages were made.

The variations observed are summarized below:—

Colonial and morphological variations of the same nature as described in the previous experiments were noted.

No change in *serological or sugar reactions* was observed.

Metabolism.—The results are given in Table III. It will be noticed that a rise in glycolysis was observed in strains Nos. 18, 20, and 22 isolated after the fourth passage, thus transferring them to Linton's metabolic group II. No. 18 was used for the next passage. The organism recovered after this passage again exhibited a low glycolytic figure. After the sixth passage, however, two strains showed some rise.

TABLE III.

Metabolic activity and chemical structure of certain strains of vibrios recovered after passage of carrier strain No. 86.

Passage number.	Strain number.	Period of isolation after feeding (hours).	METABOLISM.		Linton's metabolic group.	Chemical structure.	Linton's chemical group.
			Respiration.	Glycolysis.			
Original	86	..	5.1	2.6	III	Galactose and glucose, protein II.	Mixed IV and V.
1	1	24	6.2	3.5	III
	2	24	7.0	2.6	III
	3	24	7.2	2.8	III
	4	24	7.6	3.6	III
	5	24	6.2	3.6	III
2	6	24	7.2	3.0	III
	7	24	4.8	3.5	III
	8	24	6.8	3.2	III
	9	24	4.9	4.6	III
	10	24	6.0	3.3	III
3	11	24	10.0	3.5	V
	12	24	7.6	3.7	III
	13	24	9.3	1.9	V
	14	24	10.0	3.1	V
	15	24	8.0	4.4	II or III
4	17	24	6.4	3.8	III
	18	24	8.0	6.1	II	Galactose, protein II.	IV.
	19	24	7.5	3.3	III
	20	24	6.1	6.3	II
	21	24	7.3	3.7	III
	22	24	7.0	6.7	II

TABLE III—*concl'd.*

Passage number.	Strain number.	Period of isolation after feeding (hours).	METABOLISM.		Linton's metabolic group.	Chemical structure.	Linton's chemical group.
			Respiration.	Glycolysis.			
5	23	24	7.5	3.4	III
	24	24	9.3	0.3	IV
	25	24	8.0	2.2	III
	26	24	7.3	4.8	II
6	27	24	6.4	2.1	III
	28	24	7.6	3.2	III
	29	24	6.7	5.8	II
	30	24	6.2	4.6	II

Chemical structure.—Chemical analysis was made of strain No. 18 which showed the highest metabolic activity. The polysaccharide was found to contain pure galactose instead of a mixture of glucose and galactose of the original strain but the protein remained unchanged.

All the recovered strains were tested for the presence of bacteriophage. None was found contaminated.

D. EXPERIMENTS WITH A NON-AGGLUTINATING VIBRIO (No. 100C) ISOLATED FROM A TANK IN CALCUTTA.

The characteristics of the strain were as follows:—

Colonial appearance: Semi-opaque, smooth, entire edge.

Morphology: Curved rod.

Sugar reactions: Saccharose +, mannose —, arabinose — (Heiberg's group II).

Serological reaction: Inagglutinable against Inaba 'O' and 'H' & 'O' sera.

Metabolism: Respiration 11.4, glycolysis 1.6 (Linton's metabolic group V).

Chemical structure: Polysaccharide containing arabinose and protein II (Linton's chemical group III).

The experimental procedure was the same as in C. Altogether nine passages were made with the following results:—

Colonial and morphological changes of the same nature as described above were noted.

Sugar reaction.—Two organisms isolated in the 8th passage showed reduction of mannose also, thus becoming group I of Heiberg. But the change was transient as it was lost in the next sub-culture.

Serological reaction.—No change was observed.

Metabolism.—The results are given in Table IV. It will be seen that the glycolytic figure rose high in strain No. 108C isolated in the 8th passage and also in strains Nos. 109A and 109B isolated in the 9th passage, thus changing them into group I of Linton.

TABLE IV.

Metabolic activity and chemical structure of certain strains of vibrios recovered after passage of tank strain No. 100C through flies.

Passage number.	Strain number.	Period of isolation after feeding (hours).	METABOLISM.		Linton's metabolic group.	Chemical structure.	Linton's chemical group.
			Respiration.	Glycolysis.			
Original	100C	..	11.4	1.6	V	Arabinose, protein II.	III.
1	101A	24	12.9	0.8	IV
	101B	24	10.4	4.3	V
	101C	24	13.6	1.4	V
2	102A	24	14.5	1.1	V
	102B	24	14.3	2.6	V
	102C	24	14.0	3.9	V
3	103A	24	11.9	6.3	VI
	103B	24	11.6	6.3	VI
	103C	24	14.0	2.0	V
4	104A	24	14.5	2.8	V
	104B	24	13.4	0.9	IV
	104C	24	13.5	5.9	VI or I
5	105A	24	11.2	0.4	IV
	105B	24	12.3	4.1	V
	105C	24	14.2	4.0	V
6	106A	24	11.7	5.8	VI
	106B	24	14.4	4.4	V
	106C	24	9.3	1.3	V

TABLE IV—*concl'd.*

Passage number.	Strain number.	Period of isolation after feeding (hours).	METABOLISM.		Linton's metabolic group.	Chemical structure.	Linton's chemical group.
			Respiration.	Glycolysis.			
7	107A	24	10.7	1.4	V
	107B	24	10.2	6.7	VI
	107C	24	13.7	5.1	V
	107D	24	11.4	6.2	VI
8	108A	24	9.7	3.2	V
	108B	24	9.2	3.3	V
	108C	24	13.8	9.4	I	Galactose, protein I.	I.
	108D	24	14.2	5.4	V
9	109A	24	13.6	7.5	I
	109B	24	14.7	8.8	I

Chemical structure.—No. 108C which showed highest glycolytic figure was chemically analysed. Change in both polysaccharide and protein was observed. From polysaccharide containing arabinose it changed into one containing galactose and from protein II to protein I, thus bringing it under group I of Linton. This observation was similar to the results obtained in our previous experiment with strain No. 2252. The original as also the recovered strains did not show presence of bacteriophage.

SUMMARY.

1. Investigations have been carried out on the characters of vibrios isolated at intervals from house flies fed on cultures of vibrios of different types.

2. Certain changes in colonial characters and morphology of the strains are recorded.

3. No changes in fermentation reactions of the sugars used in Heiberg's classification were noted except a transient change in one case which was lost on subsequent sub-culture.

4. No change of 'O' serological type was recorded in the case of *V. cholerae* or other strains.

5. Changes in metabolic activity and in chemical type as determined by the methods employed by Linton and his collaborators were found in the case of many of the strains recovered from flies.

The observations given here are difficult to interpret in the light of our present knowledge of *V. cholerae*. No conclusions have, therefore, been drawn at this stage.

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OBSERVATIONS ON HÆMOLYSIS BY VIBRIOS.

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IN the present paper are recorded some of the results of investigations into the production of hæmolysins by vibrios and their properties.

STRAINS INVESTIGATED.

(1) *Basra I*.—Isolated during cholera epidemic in Mesopotamia in 1931, and received at the Central Research Institute, Kasauli, on the 27th October, 1933, through Dr. Cantacuzene. Agglutinated by serum produced with the Calcutta strain 353/3 but was inagglutinable with 'O' group I serum (Gardner and Venkatraman, 1935); belongs to Heiberg's (1935) fermentation group II; cholera-red reaction positive.

(2) *El Tor 34-D14*.—Received from Mr. Bruce White, National Institute of Medical Research, London. Corresponds serologically with Ogawa sub-type of 'O' group I. Heiberg group I. C R +.

(3) *El Tor strain*.—No information available as to source. Group VI of Gardner and Venkatraman. Heiberg group II. C R +.

(4) *Rangoon 'rough'*.—A strain isolated from a case of cholera in Rangoon on the same plate as a typical agglutinable *V. cholerae*; originally described as showing 'rough' colonial characters but now typically 'smooth' and stable in broth. It is not agglutinated by 'O' group I serum; serologically related to *V. metchnikovi*; Heiberg group II. C R +.

(5) *W 2080*.—Isolated on the 9th January, 1936, from tank water in Bengal. Inagglutinable with 'O' group I serum. Serological relationship with other strains available has not been shown. Heiberg group II. C R +.

(6) *El Tor D27*.—Received from King Institute of Preventive Medicine, Madras. Serologically identical with Ogawa sub-type of 'O' group I. Heiberg group I. C R +.

(7) *El Tor* 1 (*van Loghem*).—Received from the King Institute, Madras. Serologically identical with Ogawa sub-type of 'O' group I. Heiberg group I. C R +.

EXPERIMENTAL STUDIES.

Method employed for Quantitative Determination of Hæmolysin.

For quantitative tests dilutions were made in multiples of two in 0.85 per cent sodium chloride solution. A series of small test-tubes, 8 to 10 in number, were set up and to each tube, except the first, a volume of saline was added with a capillary pipette. One volume of the culture or filtrate to be tested was placed in the first and second tubes. The contents of the second tube were mixed by alternately drawing up and expelling a couple of times and a volume of the mixture was transferred to the third tube. This process was repeated for each tube. From the last tube of the series one volume was removed and discarded. The contents of the tubes correspond to dilutions of 1/1, 1/2 . . . 1/512. After the dilutions were made, one volume of a 5 per cent suspension of goat's washed red blood cells in saline was added to each tube. The tubes were shaken and placed in a water-bath at 37°C. for two hours after which readings were taken. The tubes were then kept in the refrigerator overnight and final readings taken in the morning. Control tubes with washed cells and saline and medium were usually put up with each series.

The Rate of Production of Hæmolysin.

Tubes of Douglas broth were inoculated with a loopful of a twenty-four culture of the various strains. The cultures were tested for hæmolysis at varying intervals of time as shown in Table I. It will be noted that the strains behaved differently. With some strains the hæmolysin could be demonstrated in the culture within the first two hours but all were hæmolytic after four hours of incubation. During the four to twenty-four hour period the hæmolysin increased rapidly and the maximum was attained between the second and third day of incubation. After remaining constant for a variable period the hæmolysin content began to fall gradually and ultimately disappeared altogether.

That the capacity of different strains to produce hæmolysin varied considerably is also shown. Some strains such as Basra I and W 2080 produced very potent hæmolysins; others were weakly hæmolytic. Strains which did not produce a powerful hæmolysin lost their hæmolytic activity earlier than those which were highly potent. Greig's (1914) observation that maximum production of hæmolysin occurs after three days' incubation is probably true of most strains, but occasionally one may come across a strain which does not show any hæmolytic action on the third day. *El Tor* 1 is an example of this type. In view of this the best procedure is to examine the culture for hæmolysis after 24 hours' incubation. If there is no hæmolysis the culture should be incubated for another 24 hours and then tested. If still negative, the test should be repeated after a further period of 24 hours' incubation.

Comparative experiments with 'rough' variants of Basra I and W 2080 have shown no significant difference in their hæmolytic power as compared with the 'smooth' parent strains. 'Rough' strains in the first few hours of incubation may be slightly weaker in hæmolysin production but ultimately show the same degree of hæmolysis as the 'smooth' ones.

TABLE I.

The effect of period of incubation on the titre of the hæmolysin produced by seven vibrio strains.

Strains.		INTERVALS OF TIME.										
		2 hours.	4 hours.	1 day.	2 days.	3 days.	4 days.	5 days.	6 days.	7 days.	9 days.	10 days.
Basra I	..	1/4	1/64	1/256	1/256	1/512	1/512	1/256	1/256	1/256	1/256	1/128
W 2080	..	—	1/8	1/256	1/256	1/64	1/64	1/64	1/64	1/64	1/16	1/16
Rangoon 'rough'	..	—	1/4	1/8	1/32	1/16	1/16	1/16	1/8	—	—	—
El Tor 34-D14	..	1/32	1/32	1/128	1/64	1/64	1/32	1/8	1/4	—	—	—
El Tor	—	1/16	1/128	1/64	1/32	1/8	1/8	1/8	1/8	1/4	—
El Tor D27	..	1/16	1/32	1/32	1/8	1/4	1/4	—	—	—	—	—
El Tor 1	..	—	1/16	1/32	1/4	—	—	—	—	—	—	—

The figures show dilutions of cultures which give definite hæmolysis.

The Lytic Action for Red Blood Cells of various Animals.

Many observers have noted that there is a marked difference in the susceptibility to hæmolysis of erythrocytes of different species of animals. The action of vibrio hæmolysin upon the red blood cells of different species of animals was tested using red cell suspensions in a concentration of 5 per cent. Table II shows the results of these titrations. The results indicate that animals could broadly be divided into three groups: (a) those whose cells are extremely susceptible to vibrio hæmolysin, e.g., guinea-pig; (b) animals whose cells are moderately susceptible to vibriolysin, e.g., goat, sheep, and rabbit; and (c) animals whose cells are much less susceptible, e.g., pigeon, monkey, man, and horse. It will further be noted from the table that there was no correlation between the cholesterol or lecithin content of the erythrocytes and their susceptibility to the action of the hæmolysin.

TABLE II.

The titre of hæmolysin of five strains of vibrios against suspensions of erythrocytes of different animal species.

Strains.	SPECIES.							
	Human.	Monkey.	Rabbit.	Guinea-pig.	Goat.	Sheep.	Horse.	Pigeon.
Basra I	1/64	1/128	1/512	1/1024	1/512	1/512	1/128	1/128
Rangoon 'rough' ..	1/4	1/8	1/16	1/128	1/16	1/32	1/4	1/8
El Tor	1/4	1/4	1/32	1/32	1/32	1/32	1/8	1/4
El Tor 34-D14 ..	1/8	1/16	1/64	1/256	1/64	1/64	1/16	1/16
W 2080	1/32	1/256	1/256	1/1024	1/256	1/256	1/64	1/64
Cholesterol content* ..	200.0	151.6	160.0	120.3	..	516.0	230.0	..
Lecithin content* ..	420.0	190.9	420.0	207.0	..	153.4	160.9	..

* These figures represent mg. per 100 c.c., *vide* Ganguly (1937).

Effect of Heat on the Hæmolysin of Vibrios.

Broth cultures of the various strains were heated at 56°C. for ten, twenty, and thirty minutes and it was found that exposure to 56°C. for ten minutes was sufficient to destroy the hæmolysin completely (Table III). Similar results were obtained with filtrates of hæmolytic cultures.

TABLE III.

Effect of heat on the hæmolysin of vibrios.

Strains.	TITRE OF HÆMOLYSIS.			
	Unheated.	HEATED AT 56°C. FOR		
		10 minutes.	20 minutes.	30 minutes.
Basra I	1/256	—	—	—
W 2080	1/64	—	—	—
Rangoon 'rough' ..	1/32	—	—	—
El Tor 34-D14 ..	1/128	—	—	—
El Tor	1/64	—	—	—

Filterability of Vibrio Hæmolysin.

The strain Basra I was grown in broth in a flask for three days at 37°C. and filtered through a Seitz filter or a Pasteur-Chamberland L₂ candle and successive 10 c.c. fractions were collected. The culture as well as the successive fractions were titrated simultaneously. In Tables IV and V are set out the results obtained.

TABLE IV.

The effect of filtration through Seitz E. K. discs of a hæmolytic broth culture upon the titre of successive fractions of 10 c.c. each.

Successive fractions.	DILUTION OF THE FILTRATE.										
	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024
Broth culture unfiltered.	++	++	++	++	++	++	++	+	+	+	-
Through Seitz, first 10 c.c.	-	-	-	-	-	-	-	-	-	-	-
Second 10 c.c. ..	±	±	-	-	-	-	-	-	-	-	-
Third 10 c.c. ..	+	±	-	-	-	-	-	-	-	-	-
Fourth 10 c.c. ..	+	+	+	±	-	-	-	-	-	-	-
Fifth 10 c.c. ..	+	+	+	+	±	-	-	-	-	-	-
Sixth 10 c.c. ..	+	+	+	+	+	±	-	-	-	-	-
Seventh 10 c.c. ..	+	+	+	+	+	±	-	-	-	-	-

Notes for Table IV and succeeding tables :—

++ = Complete hæmolysis without a trace of deposit of red blood cells.

+ = Definite hæmolysis but partial with a slight deposit of red blood cells.

± = A slight red discoloration of the supernatant fluid.

- = No hæmolysis. Complete deposition of red blood cells with a clear supernatant fluid.

TABLE V.

The effect of filtration through Pasteur-Chamberland L₃ candle of a hæmolytic culture upon the titre of successive fractions of 10 c.c. each.

Successive fractions.	DILUTION OF THE FILTRATE.										
	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024
Broth culture unfiltered.	++	++	++	++	++	++	++	+	+	+	-
Through Kieselguhr	++	++	+	+	+	+	+	-	-	-	-
Through L ₃ , first 10 c.c.	++	++	+	+	+	+	±	-	-	-	-
Second 10 c.c. ..	++	++	+	+	+	+	±	-	-	-	-
Third 10 c.c. ..	++	++	+	+	+	+	+	-	-	-	-
Fourth 10 c.c. ..	++	++	+	+	+	+	+	-	-	-	-
Fifth 10 c.c. ..	++	++	+	+	+	+	+	-	-	-	-

See notes under Table IV.

The first fraction passing through the Seitz filter was generally devoid of any hæmolytic power. The subsequent fractions showed increasing degrees of hæmolytic action but they did not equal that of the original broth culture. It will be noted that the seventh fraction showed only a titre of 1 in 16. Chopra and Roy (1938) working with vibrios and streptococci state their opinion that the hæmolysin is removed by the Seitz filter. Adsorption of the hæmolysin in some degree does occur and it is probable that if larger quantities had been filtered and if the cultures had shown a higher hæmolysin content a proportion of the hæmolysin would have passed through the filter as has been found in the experiments reported above.

When the culture was filtered through a Pasteur-Chamberland L₃ candle there was no marked difference in the hæmolytic titre of the first sample and the last, i.e., fifth sample of the filtrate. There was, however, a considerable reduction in the hæmolytic power of the broth culture as a result of filtration. With the Berkefeld N filter the hæmolytic titre was also reduced but it was noticed that these filtrates were much more potent than those either through a Seitz or a Pasteur-Chamberland filter. They were active in a dilution of 1 in 256.

Similar results were obtained with the strain W 2080.

The results show that, although definitely filterable, the hæmolysin tends to be adsorbed on the filtering disc or candle but this adsorption varies with the type of candle used, being most marked in the case of Seitz discs.

The Effect of Antiseptics on Vibrio Hæmolysin.

The action of formalin in concentrations of 0.1 per cent and 0.2 per cent and of 0.5 per cent carbolic acid on the potency of the hæmolysin was studied. Mixtures of antiseptics and filtrate (Pasteur-Chamberland L₃) of broth cultures were kept both in the incubator and in a refrigerator maintained at a temperature of 5°C. The hæmolytic action of the mixtures was tested in the usual manner at varying intervals of time. The results of one such experiment are shown in Table VI. Similar results were obtained with broth cultures.

TABLE VI.

Influence of antiseptics and temperature upon the rate of loss of hæmolysin of Basra L₃ filtrate. Original titre 1/64.

	INTERVALS OF TIME.											
	3 days.		6 days.		9 days.		13 days.		16 days.		20 days.	
	37°C.	5°C.	37°C.	5°C.	37°C.	5°C.	37°C.	5°C.	37°C.	5°C.	37°C.	5°C.
Basra L ₃ filtrate. No antiseptic.	1/32	1/32	1/8	1/32	1/8	1/32	1/4	1/32	1/4	1/32	—	1/32
Basra L ₃ filtrate + 0.1 per cent formalin.	1/4	1/32	1/2	1/16	1/2	1/16	1/2	1/16	1/2	1/16	1/2	1/16
Basra L ₃ filtrate 0.2 per cent formalin.	1/2	1/16	—	1/16	—	1/16	—	1/16	—	1/16	—	1/16
Basra L ₃ filtrate + 0.5 per cent carbolic acid.	—	1/32	—	1/32	—	1/32	—	1/32	—	1/32	—	1/32

See notes under Table IV.

It will be noticed that the addition of antiseptics did not cause much loss of the hæmolysin at low temperatures but on the other hand there was a very rapid deterioration of the hæmolysin at 37°C. The hæmolytic filtrate to which no antiseptic was added also showed a loss of its activity at 37°C. but the addition of an antiseptic caused a great acceleration of the rate of loss of the hæmolysin.

Neutralization of the Vibrio Hæmolysin by Anti-hæmolytic Sera.

The anti-hæmolysin used in the following experiments was prepared against the strain Basra I by repeated intravenous injections into rabbits of the supernatant fluid obtained by centrifuging a three days' old broth culture. The injections were made on alternate days commencing with 0.1 c.c. and ending with 1.5 c.c. Blood was taken by cardio-puncture on the seventh day after the last injection and again after a day's rest. The immunization of the same animal was continued for the purpose of obtaining further supplies of the serum.

TABLE VII.

A preliminary test to obtain the suitable range of testing the neutralizing power of the antiserum.

Basra serum dilution.			DILUTION OF BASRA I CULTURE.					
			1/1	1/2	1/4	1/8	1/16	1/32
1 in 20	—	—	—	—	—	—
1 in 25	—	—	—	—	—	—
1 in 30	+	—	—	—	—	—

See notes under Table IV.

Time allowed for antibody-antigen reaction was one hour at 37°C. in the water-bath.

TABLE VIII.

Neutralization of hæmolysin of Basra I by antiserum.

Serum dilutions.	DILUTIONS OF THE HÆMOLYTIC CULTURE.										
	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024
Control one volume of broth.	++	++	++	++	++	++	++	+	+	+	±
1 in 26 normal rabbit serum.	++	++	++	++	++	++	+	+	+	±	—
1 in 26 Basra I serum.	—	—	—	—	—	—	—	—	—	—	—
1 in 27 normal rabbit serum.	++	++	++	++	++	++	+	+	+	±	—
1 in 27 Basra I serum.	—	—	—	—	—	—	—	—
1 in 28 normal rabbit serum.	++	++	++	++	++	++	+	+	+	±	—
1 in 28 Basra I serum.	±	—	—	—	—	—	—	—	—	—	—
1 in 29 normal rabbit serum.	++	++	++	++	++	++	+	+	+	±	—
1 in 29 Basra I serum.	+	—	—	—	—	—	—	—	—	—	—
1 in 30 normal rabbit serum.	++	++	++	++	++	++	+	+	+	±	—
1 in 30 Basra I serum.	+	—	—	—	—	—	—	—	—	—	—

See notes under Table IV.

In testing the combining power of the antiserum with the hæmolysin the following method was adopted. Dilutions of the culture or filtrate to be tested were made as described in the earlier part of the paper. To each tube of the set containing a volume of the dilution of the broth culture an equal volume of a serum dilution was added. After incubation at 37°C. for one hour to allow the antibody-antigen reaction to occur one volume of 5 per cent suspension of washed goat red blood corpuscles was added to each tube. The mixtures were well shaken and then placed in the water-bath at 37°C. for two hours after which they were transferred to the refrigerator until the following morning when readings were taken. A preliminary test, as shown in Table VII, was carried out to determine the approximate range of the serum dilution which would be required to neutralize the hæmolysin of a given culture. For this purpose dilutions of sera with wide range were used. Later, a more accurate titration was made within a narrower range.

The possibility of some degree of inhibition of hæmolysis occurring due to normal serum proteins had to be considered before attempting quantitative estimation of neutralization. A preliminary test was, therefore, undertaken with normal rabbit serum on the degree of inhibition which was produced. The protective action of the normal serum was most marked with the undiluted serum and it became progressively weaker and weaker until with a dilution of 1 in 40 no marked inhibitory effect was observed (Table IX).

TABLE IX.

Showing the inhibitory effect of the normal rabbit serum on the hæmolytic action of Berkefeld filtrate of Basra I.

Rabbit serum.			DILUTION OF THE FILTRATE.							
			1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128
Control with broth	++	++	++	++	++	+	+	+
Undiluted serum	+	+	-	-	-	-	-	-
Serum diluted 1 in 5	++	++	+	+	+	-	-	-
Serum diluted 1 in 10	++	++	++	+	+	±	-	-
Serum diluted 1 in 20	++	++	++	+	+	±	-	-
Serum diluted 1 in 40	++	++	++	+	+	+	+	-

See notes under Table IV.

The allowance for inhibition by normal serum was arrived at in the following manner. For example, in one experiment (Table VIII) Basra serum diluted 1 in 27 caused total neutralization of the hæmolytic action of the culture and the m.h.d. of the culture in the presence of normal rabbit serum in a dilution of 1 in 27 was

1 in 256. Therefore, 1 in 27 antiserum neutralized 256 m.h.d. and not 512 m.h.d.: 1 in 512 being the m.h.d. of the culture in the absence of normal rabbit serum. To avoid this source of error parallel sets of the normal rabbit serum of the same dilution as the antiserum were also put up in all the neutralization experiments.

In order to determine whether the hæmolysins produced by the vibrios under investigation were homologous tests were put up in the same manner as indicated in Table VIII. The results showed that with the exception of one strain the anti-hæmolytic serum produced against Basra I neutralized the hæmolysins of other strains, namely, W 2080, El Tor, and El Tor 34-D14. The results were as follows:—

Strain.	Dilution giving definite (+) hæmolysis.	Dilution of antiserum (Basra I) required for complete neutralization of the hæmolysin.
Basra I ..	1 in 256	1 in 27
W 2080 ..	1 in 256	1 in 31
El Tor 34-D14 ..	1 in 32	1 in 225
El Tor ..	1 in 16	1 in 450
Rangoon 'rough' ..	1 in 32	1 in 74

It will be seen that the quantity of the antiserum required for neutralization varies directly with the hæmolytic titre of a culture. In other words, a strongly hæmolytic strain requires more serum for neutralization than a weakly hæmolytic strain. El Tor, for example, is a weakly hæmolytic strain which did not cause hæmolysis of the erythrocytes in a dilution higher than 1 in 16, while Basra culture is hæmolytic at a dilution (1 in 256) which is sixteen times greater and El Tor requires for the complete neutralization of its hæmolysin antiserum in a dilution of 1 in 450 which is roughly one-sixteenth of the dilution of antiserum required for the complete neutralization of the Basra hæmolysin. Similarly, El Tor 34-D14 which has a hæmolytic titre of 1 in 32, that is one-eighth of that of Basra I, is completely neutralized by a dilution of 1 in 225 which corresponds to one-eighth of the dilution required to neutralize Basra I. In the case of these strains neutralization by the antiserum of Basra I is in proportion to quantitative production of hæmolysin as judged by effective dilution. There was, however, one strain, Rangoon 'rough', which behaved differently. The hæmolytic titre of this strain was 1 in 32, i.e., one-eighth of that of Basra I. If the hæmolysin produced by this strain was exactly homologous with that of Basra I the calculated dilution of serum for neutralization would be 1 in 216 but actually it required a dilution of 1 in 74.

Greig (*loc. cit.*) in studying the neutralization of the hæmolysin of vibrios by anti-hæmolytic sera was unable to obtain definite evidence of the homologous nature of the vibrio hæmolysins. He found that whereas the serum prepared against a strain neutralized the hæmolysin of that strain completely, it had only a partial neutralizing effect on the hæmolysin of another strain. He attributed

this partial neutralization to the protective action of normal rabbit serum. It appears from a study of the protocol No. 6 of his experiments that differences in the hæmolytic titres of the strains investigated had not been taken into account in calculating the amount of serum required for the complete neutralization of their hæmolytic action. At the same time, while it appears that several of the strains now tested produce hæmolysins of homologous nature, the results with Rangoon 'rough' suggest that some strains may differ in their hæmolysins.

It may be noted that complete neutralization of the hæmolytic action of the Basra I filtrate was also obtained with anti-hæmolytic serum prepared by injections of the culture which shows that the protective action of the antiserum is not due to its interference with bacterial activity.

It may also be pointed out that a serum prepared against a typical agglutinable *V. cholerae* has no neutralizing action apart from that which cannot be attributed to the inhibitory effect of a normal rabbit serum.

Deterioration of Hæmolysin.

It was shown in an earlier part of this paper that the hæmolytic titre of a culture after reaching its maximum undergoes a gradual diminution if kept at incubator temperature. It was obviously desirable to investigate this aspect of hæmolysin more closely. A hæmolytic culture with an initial titre of 1 in 512 was divided into two parts in test-tubes, one of which was kept at 37°C. and the other in the refrigerator. The hæmolytic titre was tested at weekly intervals with the result shown in Table X:—

TABLE X.

The titre of the hæmolysin of Basra I at different temperatures and varying intervals. Initial titre of 3 days' old broth culture was 1 in 512.

Intervals of time.	TITRE OF HÆMOLYSIN.	
	37°C.	Refrigerator.
First week ..	1 in 256	1 in 512.
Second week ..	1 in 32	„
Third week ..	0	„
Fourth week ..	0	„

It will be noticed in the above table that there is a progressive reduction in the titre of vibrio hæmolysin at 37°C. Experiments were made to determine the cause of this deterioration. In view of the work of Neil and his co-worker (1926) on the oxidation and reduction of hæmolysins it appeared that the inactivity of old broth cultures of vibrios might be due to oxidation.

Experiments were first made to determine whether an inactive product could be obtained by exposing an active broth culture to air. This was done in an Erlenmeyer flask which contained a shallow layer of the active hæmolytic broth of which the titre was ascertained. This was exposed to air at 37°C. for three hours. There was no reduction in the hæmolytic power as a result of this treatment.

In another experiment oxidation of the hæmolysin was attempted by blowing air into a tube of broth culture through a sterile pipette plugged with cotton-wool for half an hour but without any effect.

In order to determine whether deterioration of hæmotoxin could be inhibited by lack of oxygen the following experiment was made. A hæmolytic culture with a titre of 1 in 1,024 was placed in glass ampoules which were filled to the neck leaving as little air as possible. One of the ampoules was kept at 37°C.; the other in the refrigerator. After two months' storage the ampoule kept in the incubator was found to be devoid of any hæmolytic activity, whereas the other stored in the refrigerator was active in a dilution of 1 in 512.

Experiments were then made to determine whether the inactive product obtained by allowing the culture to age in the incubator could be re-activated by the action of reducing agents as has been done in the case of hæmolysins of pneumococcus, streptococcus, etc. A hæmolytic culture with an initial titre of 1 in 512 was kept in the incubator at 37°C. On the 20th day when it showed no hæmolysis even undiluted, 5 milligrams of sodium hydrosulphite were added to 5 c.c. of the broth and covered with vaseline and incubated at 37°C. This method of treatment had no effect in restoring hæmolytic activity.

The experiments described above make it clear that the deterioration of the hæmolysin of vibrios is not dependent upon its oxidation as is the case with hæmotoxins of other organisms such as *pneumococcus*, *streptococcus*, etc., and that the inactive vibrio culture cannot be re-activated by the action of reducing agents.

DISCUSSION.

It is important to consider the question of the nature of hæmolysins of vibrios. A distinction is usually drawn between the lysis of red blood cells caused by bacterial lysins which exhibit antigenic properties and that caused by other substances of bacterial origin which are not antigenic. The latter may be of various kinds. They may be of the nature of enzymes, e.g., Orcutt and Howe (1922) isolated from milk a streptococcus the hæmolytic action of which was found to be due to the activity of a fatty acid and soap formed by a lipase acting upon the fat contained in the medium. Other substances such as acids produced during the growth of an organism may have a similar action. A bacterial hæmotoxin is a hæmolysin which resembles a true toxin in that it (1) is thermolabile, (2) is antigenic, and (3) is filterable. Greig

(*loc. cit.*) in applying these criteria to the hæmolysins of the cholera-like vibrios did not arrive at any definite conclusion. The results of experiments presented in this paper indicate that vibrio hæmolysin is an exotoxin. It is found in the fluid part of young broth cultures and does not appear to result from autolysis. Furthermore, these hæmolysins are filterable though it was noticed that there was a certain amount of adsorption during the process of filtration. The vibrio hæmolysin was found to be antigenic, that is to say, a hæmolytic culture on parenteral injection gave rise to antibodies in the serum of an animal which were capable of neutralizing the hæmolysin *in vitro*. It has also been shown that the vibrio hæmolysin is extremely sensitive to heat. It will thus be seen that the hæmolysin of vibrios fulfils all the criteria of an exotoxin and, therefore, must be regarded as a true exotoxin.

SUMMARY.

1. The rate of production of hæmolysin by vibrios varies with different strains. It has been confirmed, as found by Greig, that maximum production of hæmolysis occurs after three days' incubation with most strains but the existence of weakly hæmolytic strains which lose their hæmolysin during this period of incubation makes it necessary that hæmolytic tests should be performed at successive 24-hours intervals.

2. The red blood cells of different species of animals show great differences in their sensitiveness to the hæmolysin, those of the guinea-pig being most sensitive. The red blood cells of goat, sheep, and rabbit were next in order, and were all three susceptible in about the same degree. The cells of the pigeon, monkey, horse, and man were much less susceptible. There does not appear to be any correlation between the cholesterol or lecithin content of the cells and their sensitiveness to the action of hæmolysin.

3. The vibrio hæmolysin was found to be filterable, the most potent filtrate being obtained through a Berkefeld filter. It was also filterable through Pasteur-Chamberland L₃ candles and Seitz E. K. discs. A certain amount of adsorption occurred on all these filters and it was necessary to pass considerable quantities of culture through in order to obtain an active filtrate.

4. The hæmolysin of vibrios was found to be completely destroyed both in culture and filtrates by heating at 56°C. for ten minutes.

5. Hæmolytic action is rapidly lost if cultures or filtrates are kept at 37°C. The resulting deterioration has not been found to be due to oxidation as in the case of *pneumococcus* and *streptococcus* hæmolysins.

6. The hæmolysin of filtrates and cultures remains active for prolonged periods when kept in cold storage with or without the addition of antiseptics such as 0.5 per cent carbolic acid and 0.2 per cent formalin.

7. It has been found that quantitative cross neutralization of the hæmolysin of different strains with an immune serum prepared against a hæmolytic strain is fairly constant. The results with the strains tested indicated that the hæmolysins they produced were of homologous nature. Slight quantitative differences were

shown. Immune sera prepared against non-hæmolytic strains (typical *V. cholerae*) did not show this neutralizing action.

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OXIDATION-REDUCTION POTENTIALS OF *VIBRIO CHOLERÆ* AND RELATED ORGANISMS.

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BACTERIA in general derive the energy required for their growth and multiplication from the medium constituents which in their turn are reduced. As a result there are set up definite electrode potentials in cultures, although the exact mechanism of such electrode potentials is yet unknown. Many references will now be found on the subject of oxidation-reduction potentials of different bacterial systems. Tuttle and Huddleson (1934) reported on the oxidation-reduction potentials of the *Brucella* strains where they failed to distinguish the three types of *Brucella* by their potential curves in liver infusion broth. But they did observe a marked difference among them when bacteriostatic dyes were added to the medium. Burrows and Jordan (1935) substantiated the serological classification of the *Salmonella* group by their studies on oxidation-reduction potentials. They showed that the potential curves of the various species of the *Salmonella* group when grown in nutrient broth are significantly different from one another and are constant for each species. These were *S. cholerae suis*, *S. paratyphi A*, *S. typhi*, *S. paratyphi B*, *S. Schottmüller*, and *S. enteridis*.

Linton and his collaborators have published a series of papers on the classification of cholera organisms and related species on the basis of their chemical composition and also on their metabolic activities. It was thought desirable to work on the oxidation-reduction potentials of these organisms, which are at the basis of their synthesis, and the present paper is an account of the work done on that line.

MATERIALS AND METHODS.

The instrument employed for measuring the electrode potentials and pH values of the cultures is a Cambridge electrometre valve combined potentiometre and pH metre, and is very convenient for the purpose. The elaborate arrangement of the

diverse parts, such as potentiometre, galvanometre, electrometre, standard cell, etc., needed for the work of this kind are all packed in a small box with proper connections, thereby greatly simplifying the measurements of potentials and pH values. The readings are given in millivolts and are correct to ± 0.01 mvt.

One c.c. of 18 hours' peptone-water culture of the organism was inoculated into 100 c.c. of 1 per cent peptone water buffered with a phosphate at a pH of 7.6 to 7.8 and contained in a 200-c.c. lipless beaker. The beaker was closed with a tight-fitting rubber cork having four holes in it. Platinum wires were sealed to the ends of two narrow-bored glass tubes which were passed separately through two holes to form two separate electrodes. The contact was made with mercury filling the glass tubes. Through another hole was passed a small tube into which had been blown a perforated bulb which served as an outlet for gases that might be formed in the culture. An agar bridge was fitted into the fourth hole. All necessary connections for readings were subsequently made and precautions were taken at all stages against contamination. The arrangement of the entire operation has been shown in Plate XI. The organisms were placed in an incubator at 37°C. and periodic observations were made with the two platinum electrodes separately. Simultaneously, the test organisms were grown in similar media and the changes in the pH values were observed at definite intervals as the organisms grew, by means of quinhydrone electrode in the same apparatus.

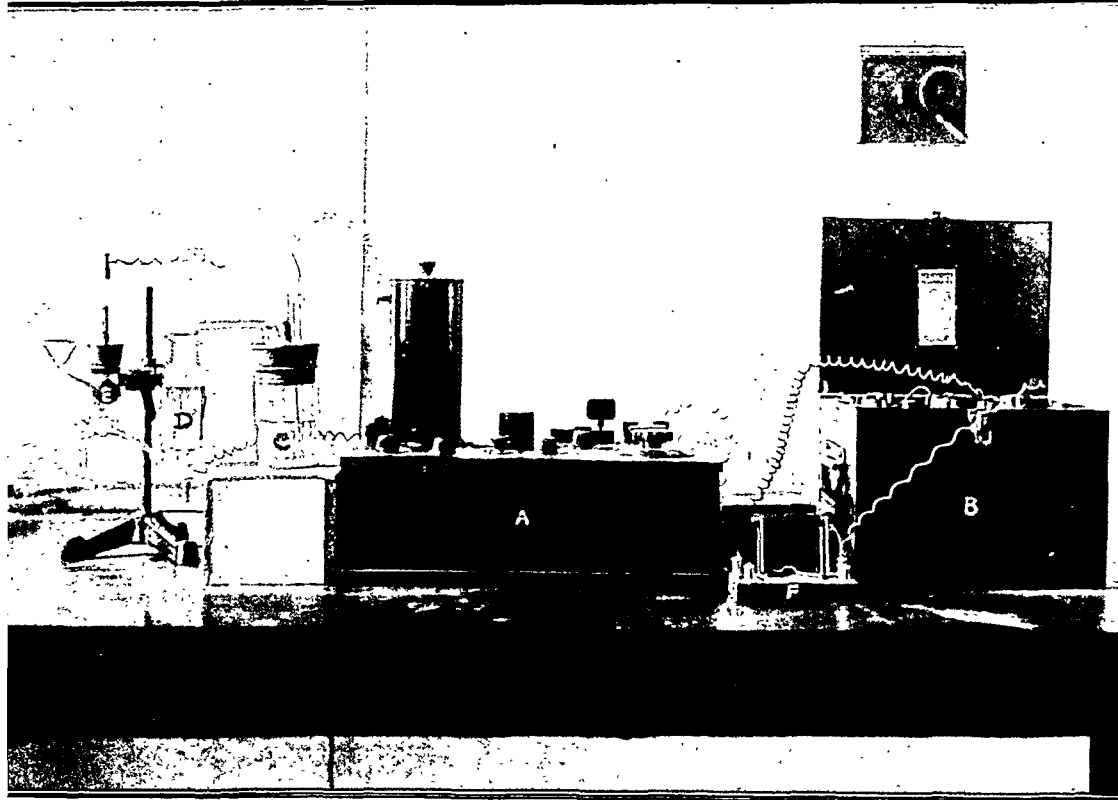
EXPERIMENTAL.

In all, 36 vibrio strains derived from various sources and composed of six strains from each of the six chemical groups were examined. It was found that initially the potentials of all the strains were more or less the same. In about six hours the potential fell down to the minimum and was different in different groups but was very nearly the same for the strains of each individual group. After six hours of incubation the potential began to rise gradually till it reached its maximum in about thirty hours, and thereafter the potential usually remained stationary up to a period of 76 hours when the experiment was terminated. It should, however, be mentioned that after the potential had reached the minimum level some fluctuations were observed in the potentials of several strains. The lower and upper limits of the end-points of the curves reached by the strains of each group were as follows:—

Chemical group.			Range of E_h in millivolts.
Protein I	I	..	+ 400 to + 450.
	II	..	+ 350 to + 400.
	VI	..	+ 300 to + 400.
Protein II	IV	..	+ 250 to + 300.
	III	..	+ 200 to + 300.
	V	..	+ 200 to + 250.

PLATE XI.

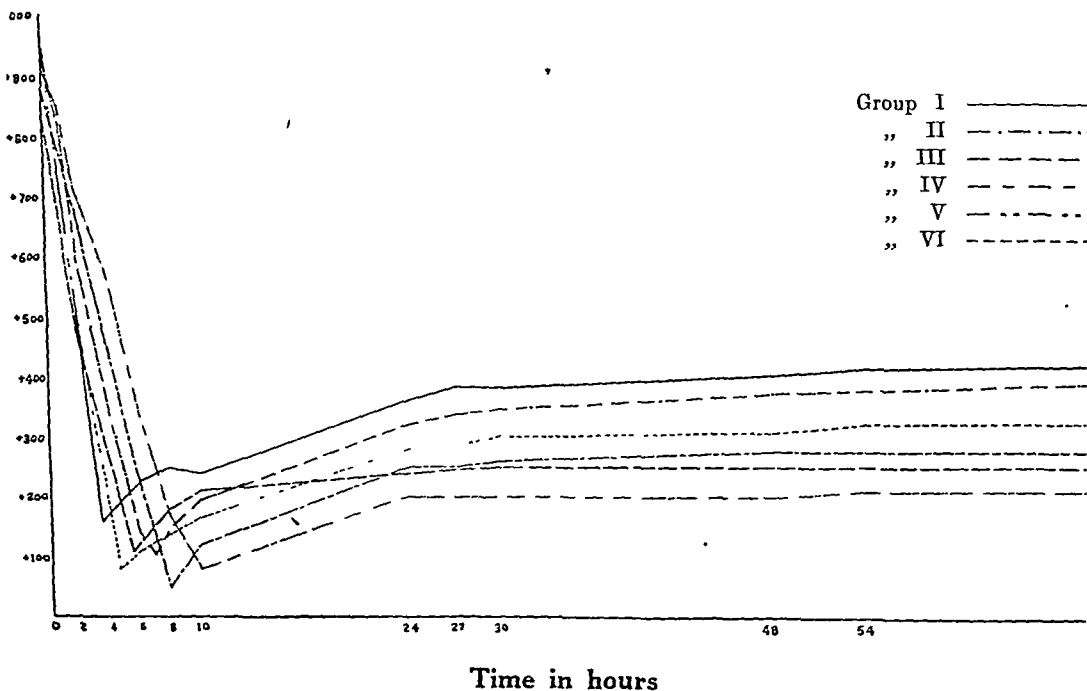
Showing the potentiometre in operation.



- A. Cambridge electrometre valve combined potentiometre and pH metre.
- B. Battery.
- C. Culture beaker fitted with duplicate electrodes, agar bridge, etc.
- D. Calomel glass electrode.
- E. Hydrogen glass electrode.
- F. Commutator.

From the above figures it is apparent that the potential curves are distributed well over a range, thereby rendering it difficult to differentiate the vibrio strains from one another. But when the average curves of the strains belonging to different chemical groups are plotted they are certainly different from one another as will be seen from Graph 1. It is also interesting to note that the range of end potential exhibited by the strains of groups I, II, and VI is from 300 to 450 millivolts and is higher than that shown by the strains of groups III, IV, and V, which is from 200 to 300 millivolts. Vibrio strains belonging to the former three groups are characterized by their possession of protein I, whereas those belonging to the latter three have protein II. The two vibrio proteins have been shown to be structurally different (Linton, Mitra and Shrivastava, 1934; Mitra, 1936) and this fact has been further corroborated by the present observation on oxidation-reduction potentials.

GRAPH 1.



Showing the relation of the characteristic oxidation-reduction potentials of six chemical groups of vibrio

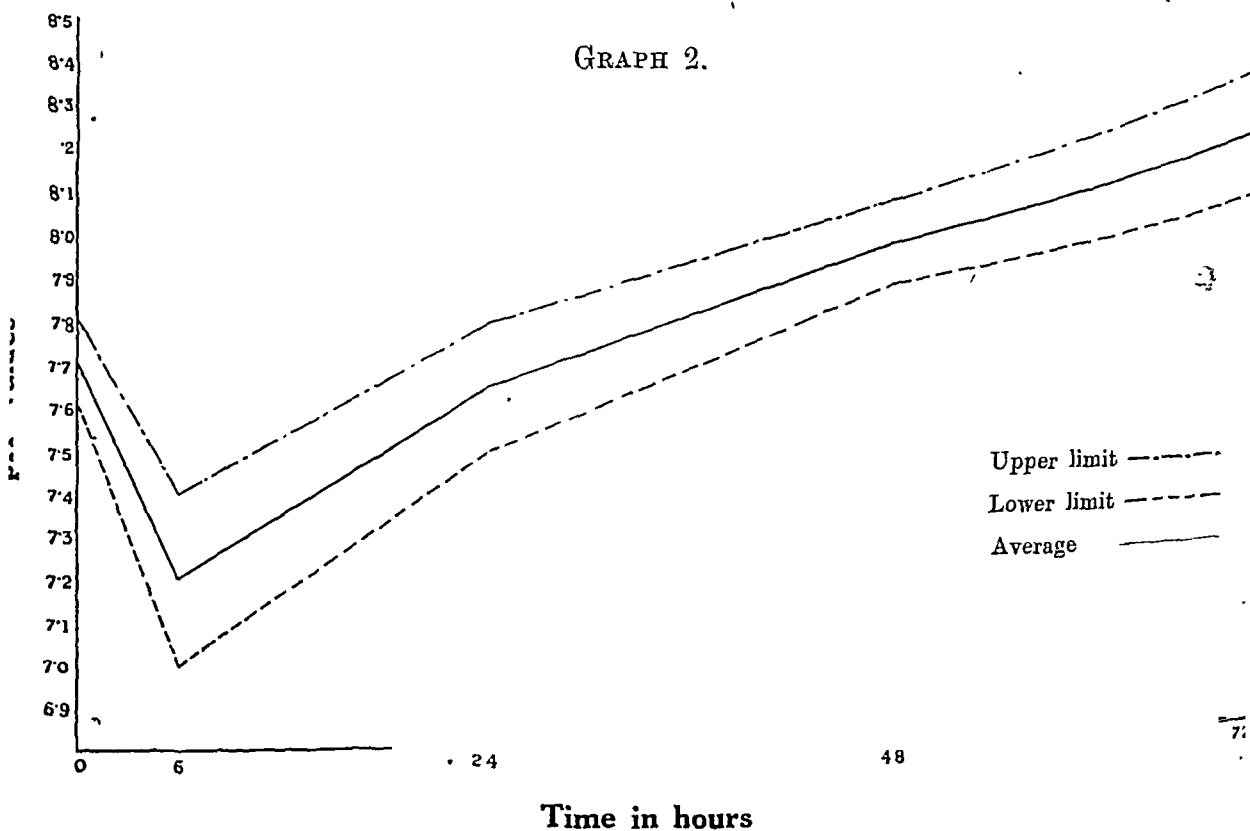
The average potential curves seem to indicate that the vibrio strains of different chemical groups are different, and are consistent with the fact that the vibrios have different metabolic activities (Linton, Mitra and Mullick, 1936a and b). It is well known that the shape of a potential curve may be greatly modified by changing the medium or by introducing different dyes in the same substratum, so that by using different media other than that used it may be possible to effect a marked change in the electrode potentials. Further work on this line is therefore necessary.

Another point which emerges out of the study of the graph is that soon after inoculation the potential commences to fall and a minimum value is reached in about six hours; this is apparently the so-called lag phase of the growth of the organisms under the conditions of the experiment. During the process of metabolism after this period the potential gradually rises corresponding to the logarithmic phase when the organisms begin to multiply and in about thirty hours' time they reach about a stationary level, indicating the cessation of active multiplication. This also suggests that to get the maximum amount of growth of a vibrio culture in 1 per cent peptone water 30 hours' growth is quite sufficient for the purpose.

The observations made on changes in pH in vibrio cultures in 1 per cent peptone water are given below. The range of variation of pH given in the table shows the lower and higher limits indicated by some of the organisms irrespective of their chemical groups.

	Initial.	6 hours.	24 hours.	48 hours.	72 hours.
pH figures ..	7.6—7.8	7.0—7.4	7.5—7.8	7.9—8.1	8.1—8.4

The change in pH values offers an interesting study. Although all the organisms do not have the same change in pH, the general trend of fall and rise is,



Showing the general trend of variation of pH in vibrio cultures.

however, similar in all cases. There is a definite fall in about six hours' time and then a gradual rise up to 72 hours, after which no further readings were taken. This has been graphically represented in Graph 2 showing three curves, viz., the lower limit, the upper limit, and the average curves.

It is interesting to note that the pH curves have a great similarity with the potential curves except that the latter almost cease to rise after 30 hours, whereas the former slowly rise till at least 72 hours. Another point of difference is that, while the potential curves are different for vibrios belonging to the different chemical groups, such is not the case with the pH curves.

It is now known that the lag phase of an organism is the one when the organism is at its maximum of metabolic activities (Topley and Wilson, 1936) and thus corresponds to the minimum electrode potential and fall in pH. After this period when the organisms have fully adapted to the medium and environment including the pH, they begin to multiply and are attended with a gradual increase in pH as well as in potential. -

SUMMARY AND CONCLUSION.

1. The electrode potentials of vibrio cultures in 1 per cent peptone water buffered with a phosphate at pH 7.6 to 7.8 were measured by means of a Cambridge electrometre valve combined potentiometre and pH metre.

2. The potential curves of the organisms studied for each individual chemical group were more or less distributed over a range. The average potential curves of the organisms belonging to different chemical groups were, on the other hand, different from one another.

3. Organisms containing protein I usually had higher electrode potentials than those containing protein II.

4. Under the conditions of the experiment the electrode potentials fall down to minimum in about six hours and then gradually rise up reaching the maximum in about thirty hours.

5. The fall in electrode potentials corresponds to the so-called lag phase of the growth of the organisms and is characterized by having low pH in the medium.

6. The pH values rise along with the increase in potentials and unlike the latter affect all organisms in a general way irrespective of their chemical groups.

ACKNOWLEDGMENT.

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CALORIE REQUIREMENTS OF SOUTH INDIAN CHILDREN.

BY

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THE human organism requires daily a certain quantity of food to support bodily activity, the quantity required being dependent on such factors as age, sex, climate, and the amount of work done. The problem of establishing satisfactory average standards of calorie standards, applicable to groups, has received much attention. As far as Western populations are concerned, the standards suggested by the League of Nations' Technical Commission on Nutrition (1936), which apply to the various age and sex groups and indicate the corrections to be made according to the amount of work performed, may presumably be accepted as satisfactory for practical nutrition work. It is, however, doubtful whether the League of Nations' standards can be adopted without question by the nutrition worker in the East, who is concerned with populations of a different type living under different conditions.

Aykroyd and Krishnan (1937) suggested that the minimum daily calorie requirements of a South Indian peasant are in the neighbourhood of 2,500. In Health Bulletin No. 23 (1936), the minimum calorie needs of an average Indian man, 'engaged in ordinary easy-going agricultural or coolie work', are assessed at 2,500 to 2,600 calories per diem. These figures are some 10 per cent below the League of Nations' standard for a man engaged in 'moderate' work. They refer to 'food actually eaten', the calorie value of which is calculated from the tables given in the Health Bulletin.

In order to carry out diet surveys in which the food intake of families of varying age and sex composition must be compared, it is necessary to use a scale of co-efficients in which the energy requirements of women and children of various age and sex are stated in terms of those of an adult man. Estimates of the calorie needs of children are also necessary for preparing diet schedules for children's institutions. For these purposes the Nutrition Research

Laboratories have so far employed the scale recommended by the League of Nations' Health Organization (1932), which is given below :—

Age.	Male.	Co-efficient, both sexes.	Female.
Over 60	0·8	..
14 to 59 ..	1·0	..	0·8
12 and 13	0·8	..
10 „ 11	0·7	..
8 „ 9	0·6	..
6 „ 7	0·5	..
4 „ 5	0·4	..
2 „ 3	0·3	..
0 to 2	0·2	..

These co-efficients are adopted in Health Bulletin No. 23 for estimating calorie requirements, the figure 2,600 representing the needs of an adult man. The resulting scale is as follows :—

Adult male (over 14) ..	2,600
„ female „ ..	2,080
Child 12 and 13 years ..	2,080
„ 10 „ 11 „ ..	1,820
„ 8 „ 9 „ ..	1,560
„ 6 „ 7 „ ..	1,300
„ 4 „ 5 „ ..	1,040
„ 2 „ 3 „ ..	780
„ 0 to 2 „ ..	520

THE ACTUAL CALORIE INTAKE OF CHILDREN.

It has been fully recognized that the above co-efficients and calorie equivalents are conventional and may differ from actual intake in certain age groups. To throw light on this question, an investigation to determine food intake at different age levels was undertaken. A boys' boarding school in the south of the Madras Presidency, containing about 150 children, was chosen. All the children were

examined, and none showed any signs of deficiency disease. They appeared to be a healthy and active group of children on the whole, and the medical authorities of the school were satisfied with their general health. The diet was vegetarian. The staple cereal was rice, which was either lightly milled or home-pounded. Ragi was usually consumed once a day. The supply of fresh vegetables, fruit, and milk appeared adequate. The children were divided into five groups corresponding to five age periods of the above scale, all the available children being included. The children below 2 could obviously not be included in the investigation, and the ages of children above 11 could not be verified with any accuracy. The five groups consisted of children of the following ages:—

1. Two and three.
2. Four and five.
3. Six and seven.
4. Eight and nine.
5. Ten and eleven.

The following method was adopted to determine calorie intake in the various groups. Special kitchen arrangements were made so that the food of each group was kept and prepared separately. A record of raw food issued to each of the groups was made, and the raw food was weighed before cooking. The children were fed under the direct supervision of the author and were allowed to consume as much as they wanted. Any food left over was used the next day by the same group and thus no food issued was thrown out and wasted. The children belonging to the various groups were not allowed to mix with one another during meals and no interchange of foodstuffs was allowed. This arrangement continued for a week. On the last day of the week, any cooked food left over was weighed. Total intake per day in each of the groups was calculated and this was divided by the number of children in each group to give the average daily consumption per head.

Table I shows calorie intake as worked out from data given in Health Bulletin No. 23. Figures for intake of proximate principles, calcium, and phosphorus are also included.

TABLE I.

Per capita intake of calories, etc., in the various groups.

Age.	Number of children.	Calories.	Protein.	Fat.	Carbo-hydrate.	Calcium.	Phosphorus.
2 and 3 ..	17	870	32	32	109	0.89	0.81
4 „ 5 ..	18	1,140	38	42	151	0.92	0.95
6 „ 7 ..	18	1,300	44	39	190	1.08	1.06
8 „ 9 ..	29	1,600	42	28	293	0.95	0.99
10 „ 11 ..	25	1,760	46	29	326	0.98	1.07

In Table II observed calorie intake is compared with the Health Bulletin standards.

TABLE II.

Observed calorie intake compared with standard.

Age group.	H. B. standard.	Actual intake.	Difference, per cent.
2 and 3 ..	780	870	+ 11
4 „ 5 ..	1,040	1,140	+ 10
6 „ 7 ..	1,300	1,300	<i>Nil.</i>
8 „ 9 ..	1,560	1,600	+ 3
10 „ 11 ..	1,820	1,760	- 3

In general the observed figures for energy intake correspond well with those given in Health Bulletin No. 23 for ages above 6. As regards younger age groups, it would be preferable to assign a co-efficient of 0.35 for 2 and 3, and 0.45 for 4 and 5. The present investigation, however, suggests that the scale of calorie requirements and consumption co-efficients now in use is sufficiently accurate for practical purposes,—i.e., for calculating food intake per consumption unit or adult man in dietary surveys and for drawing up dietary schedules—, and its continued use may be recommended. The investigation reported here deals only with boys, while in the scale of co-efficients at present in use the energy needs of boys and girls are assessed at the same figure. A study of the intake of girls of various ages is therefore desirable.

DISCUSSION.

Calorie intake figures given in this paper are based on the calorie value of the edible portion of raw foods as listed in Health Bulletin No. 23. The Bulletin values were obtained from the chemical determination of protein and fat content, carbohydrate content being calculated by difference. For this purpose the following co-efficients were employed :—

	Calories.
One gramme protein	= 4
„ „ carbohydrate	= 4
„ „ fat	= 9

These are sometimes known as Atwater's co-efficients and represent a modification of Rubner's co-efficients (4.1 ; 4.1 ; and 9.3, respectively). They are designed

to allow for the fact that neither the digestion of foods nor their subsequent assimilation is accomplished with complete theoretical efficiency. Bigwood (1938) states that 'by applying these co-efficients to the quantities of energy-bearing principles contained in the food *actually ingested*, as calculable in individual or family-cum-individual weighing investigations, it is possible to obtain a true assessment, requiring no further modification, of the physiologically effective heat, i.e., of the *net* calories in the diet'.

In family diet surveys weighings are carried out on uncooked food about to be eaten. The Health Bulletin analyses were made on the 'edible portion' of foodstuffs. The foodstuffs which form the bulk of the Indian diet (e.g., cereals and pulses) contain little or no 'inedible' material. Further, the poverty of the country ensures that the 'inedible' portion of foodstuffs in common use is reduced to a minimum. If, therefore, the results of a diet survey show that calorie intake, based on the weighing of raw foods about to be consumed, is equivalent to the Health Bulletin standards, this intake can probably be deemed sufficient. The standards are indeed adjusted for application on this basis. In drawing up schedules for institutions, on the other hand, it is advisable, as the Bulletin states, to 'err on the side of excess by 100 to 150 calories to allow for waste of all kinds, including the inevitable "leakage" of food which occurs in large institutions'. The same considerations apply in the calculation of calorie intake from tables of food purchased or consumed as supplied by institutions. An excess of about 5 per cent over standard requirements can be considered satisfactory. A similar allowance may be made for waste in family budget inquiries in which the physiological value of diets is calculated from expenditure on foodstuffs.

Nicholls (1938) adopts the Health Bulletin standards, but goes on to say that 'these represent the values of the food assimilated. In practice, however, it is usually necessary to calculate the calories of the foodstuffs as purchased and an allowance of about 20 per cent must be made for wastages, such as the trimmings and bones of meat and fish and the skins of tubers, which occur during the preparation and cooking of the foodstuffs'.

An allowance of 20 per cent for 'waste' of this kind seems altogether excessive.

SUMMARY.

1. An investigation of the energy intake of boys belonging to five age groups was carried out with the ultimate object of establishing a suitable scale of calorie requirements and food consumption co-efficients for children of different ages in South India.

2. There was fair correspondence between observed consumption and the scale given in Health Bulletin No. 23, which is now being widely used in nutrition work, including diet surveys, in India. The continued use of this scale, along the lines indicated in the Bulletin, is recommended.

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AVAILABLE CARBOHYDRATE AND ACID-BASE BALANCE IN PULSES.

BY

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PULSE proteins have been the subject of thorough and systematic investigation in these laboratories. Basu *et al.* (1936a and b, 1937) have determined the biological value of the pulse proteins both by the balance-sheet and by the growth methods. Basu, Nath, Ghani and Mukherjee (1937) have also extracted and analysed the proteins. These studies have revealed differences not only in protein contents but also in the biological value and the amino-acid make-up of the proteins of the pulses.

Very little information is available regarding the carbohydrate and minerals in pulses. All available food-tables give carbohydrate values which have been obtained by difference and which, therefore, must include such unavailable carbohydrate as is present. "Many green vegetables like salad, lettuce and spinach contain hardly any carbohydrate other than their cellulose of the cell-membranes" (McCance and Lawrence, 1929). No direct determination of the available carbohydrates of the pulses is on record. The need for precise knowledge of the carbohydrate content of food has been apparent in recent years because of the necessity of preparing accurate food-tables for diabetic diets. Available carbohydrate consists of starch and the soluble sugars, sucrose, glucose, and fructose, the unavailable mainly of hemicelluloses and fibre (cellulose). The

possible value of pentoses and pentosans as sources of energy is doubtful. Figures for available carbohydrates of the fruits and vegetables commonly eaten in the United Kingdom were given by McCance and Lawrence (*loc. cit.*). Their figures in some instances are rather too low because of the strong acid employed and because the prolonged time of boiling led to destruction of a considerable portion of fructose. Very few analyses of the carbohydrate content of cooked foods are available and the content of available carbohydrate of many foods is considerably altered by cooking.

The importance of the mineral matter in foods is being realized nowadays. Not only is the quantity of the ash constituents of significance, but, as Sherman and Gettler (1912) have shown, the acid- or base-forming potency of ash of different foods is also important. It has been demonstrated by Sherman and Gettler (*loc. cit.*) that vegetables and fruits, on burning, leave an ash in which the basic elements (sodium, potassium, calcium, and magnesium) predominate and they are regarded as foods of decided potential alkalinity; whereas meats of all kind, eggs, and fishes leave an ash in which the acid-forming elements (chlorine, sulphur, and phosphorus) predominate. The breadstuffs and cereals show a slight potential acidity, while cream, milk, and milk products show a slight potential alkalinity.

Many fruits and fruit juices contain acid salts of organic acids. These foods as eaten have an acid reaction but so far as the balance of non-volatile acids and bases and their influences on the urine is concerned, these are potentially base-forming foods.

"Urinary kidney-stones" often contain uric-acid crystals in considerable quantities. The human organism cannot destroy uric acid but can only transport and excrete it through the kidney. The more acid urine possesses decreased solvent power for uric acid. The reduction in the hydrogen-ion concentration of the urine may be secured by feeding properly selected base-forming foods. The benefit of health which so generally results from the use of diets consisting largely of fruits, vegetables, and milk may perhaps be attributed in part to the fact that these foods yield alkaline residues when oxidized in the body; but there are several other ways in which the eating of liberal amounts of these foods is beneficial, notably by enriching the diet in calcium, phosphorus, iron, and vitamins.

"Such considerations tend to lend weight to the view that there may be some merit in furnishing a sufficient quota of fixed-base-forming elements to balance the fixed-acid-forming elements of the intake" (Sherman, 1935).

There are many ways in which such a balance between acid-forming and base-forming elements may be constructed and stated quantitatively. Probably the clearest and most familiar is to count phosphoric acid as functioning in this connection as a diacidic acid, the acids and bases corresponding to chlorine, sulphur, sodium, potassium, calcium, and magnesium being given their ordinary stoichiometric values. The balance of acid-forming and base-forming elements in the foods is clearly indicated and given a convenient form of numerical expression, if we calculate, as suggested by Sherman, the equivalent in normal acid of the chlorine, phosphorus, and sulphur present, the equivalent in normal alkali of

calcium, magnesium, sodium, and potassium and obtain by difference the excess of acid or base as the case may be. This may be expressed in terms of cubic centimetres of normal acid or normal base for any given quantity of food material.

The pulses form a daily constituent of Indian diets. It is, therefore, advisable to determine the potential acid-forming capacity of the different pulses.

EXPERIMENTAL.

The following pulses were investigated :—

Green gram (*Phaseolus mungo*), field pea (*Pisum sativum*), lentil (*Lens esculenta*), Bengal gram (*Cicer arietinum*), khesari (*Lathyrus sativum*), soya bean (*Glycine hispida*), and arhar (*Cajanus indicus*).

Available carbohydrate.—Sucrose, glucose, fructose, and starch have each been determined separately. Their sum constitutes the available carbohydrates of the pulses. Small amounts of maltose may be present along with starch, but the error from this would be insignificant (Widdowson and McCance, 1935).

METHODS.

Pulses were obtained from the local market. Particular attention was given to securing pure samples. The outer coatings were removed and the edible portion dried to constant weight. The method followed was mainly that of Widdowson and McCance (*loc. cit.*) and is outlined below.

Duplicate portions of 100 g. were extracted with about 200 ml. of cold 95 per cent alcohol and kept overnight. The extract was filtered and the residue was again extracted with hot 80 per cent alcohol for about 16 hours. The alcohol from the united extracts was evaporated under reduced pressure at a temperature always below 30°C. The residue was made up to 250 c.c. in a graduated flask (solution A).

DETERMINATIONS OF REDUCING SUGARS.

Fifty c.c. of solution A were measured into a graduated 500-c.c. flask, diluted with water, almost neutralized with N/10 NaOH and cleared with basic lead acetate and saturated sodium phosphate solution (Archbold and Widdowson, 1931). The solution was made up to 500 c.c. and filtered (solution B). Reducing sugars were determined in this cleared filtrate by Shaffer and Hartmann's (1921) method. Dilution of the solution B was sometimes necessary before the estimations could be carried out.

Widdowson and McCance (*loc. cit.*) in their investigation of available carbohydrate of fruits used Lane and Eynon's (1923) copper-titration method with methylene blue as an internal indicator, for determining reducing sugars. But in our trial experiments with standard substances we found that Shaffer and Hartmann's method gave more accurate and concordant results and much sharper end-points with less effort than the method of Lane and Eynon.

TABLE I.

Name of pulse.	Moisture.	Protein.	Fat.	Ash.	Fibre.	Glucose.	Fructose.	Sucrose.	Starch.	Total available carbohydrate.	Total carbohydrate by difference.
Bengal gram (<i>Cicer arteinum</i>).	15.11	23.87	5.75	2.59	3.92	0.073	0.112	0.172	35.890	$\begin{matrix} = (6 + 7 + \\ 8 + 9). \end{matrix}$ 36.247	$\begin{matrix} 100 - (1 + 2 + \\ 3 + 4 + 5). \end{matrix}$ 48.76
Green gram (<i>Phaseolus mungo</i>).	15.00	23.26	2.88	3.39	0.81	0.357	0.518	0.922	47.27	49.067	54.66
Lentil (<i>Lens esculenta</i>)	15.14	22.60	2.09	1.78	0.86	0.080	0.212	1.761	50.00	52.053	57.53
Khesari (<i>Lathyrus sativa</i>).	10.1	32.20	2.9	2.1	0.90	0.085	0.171	0.519	50.145	50.920	51.80
Field pea (<i>Pisum sativum</i>).	11.5	27.10	2.10	3.20	0.80	0.520	0.890	3.356	43.015	47.781	55.30
Soya bean (<i>Glycine hispida</i>).	11.50	41.20	17.50	4.10	4.20	0.115	0.179	1.016	6.775	8.085	21.50
Arhar (<i>Cajanus indicus</i>)	12.18	24.19	1.13	3.28	3.60	0.135	0.274	0.409	49.840	50.658	55.62
Black gram (<i>Phaseolus mungo</i> var. <i>Linn</i>).	10.90	25.19	2.29	3.39	0.91	0.127	0.179	0.150	41.150	41.606	57.32

Determination of sucrose.—Total sugar was estimated in solution B after treating it with 10 per cent citric acid at 100°C. The difference between the percentages of total sugar and reducing sugar gave the percentage of sucrose in the pulse.

Determination of the fructose/glucose ratio.—Before carrying out the iodometric procedure for the determination of the fructose/glucose ratio, the solution B was decolorized. Fifty c.c. were boiled with 0.5 g. B.D.H. animal charcoal for one minute and filtered. This procedure was repeated until the liquid was colourless. The charcoal was thoroughly washed with boiling water and filtrate and washings were made up to 100 c.c. (solution C). It has been shown by Archbold and Widdowson that this procedure removed only negligible amounts of sugar from the solution. The iodine value of solution was determined exactly as described by Archbold and Widdowson. The fructose/glucose ratio was calculated by solving the simultaneous equations obtained from iodometric determination and from the estimation of the reducing power of solution B by Shaffer and Hartmann's method.

Determination of starch.—The residue after extraction with alcohol was carefully collected and dried to constant weight. A small portion was tested with iodine and the presence of starch was determined. Duplicate portions of 0.5 g. were treated with water and taka-diastase for about 12 hours, and a few drops were tested with iodine to ascertain the completion of the hydrolysis. The solution after hydrolysis was diluted, cleared with basic lead acetate, filtered, and the reducing power determined on an aliquot portion of the filtrate by Shaffer and Hartmann's method. The determination of starch can also be carried out on the clear filtrate by Hanes's (1929) modification of the Hagedorn and Jensen ferricyanide technique.

The results of analysis of the pulses are recorded in Table I. The figures given represent percentages.

DISCUSSION.

The determination of available carbohydrate by chemical methods showed that the availability in cases of green gram, lentil, khesari, field pea, and arhar was over 90 per cent. Bengal gram and black gram gave lower values showing that quite an appreciable amount of carbohydrate is unavailable. The availability of soya bean carbohydrate was found to be only 37 per cent. Recently, Adolph and Kao (1934) have published a paper on the biological availability of soya bean carbohydrate. In the *in vitro* method they determined the total reducing sugars by hydrolysis with taka-diastase and found that 27.1 per cent of the total carbohydrate in the whole soya bean was digested. Their result obtained by the determination of extra glucose in phlorhizinized rats gave a mean value of 38.5 per cent.

ACID-BASE BALANCE.

The minerals were estimated by the methods recommended by A. O. A. C.

Table II gives the percentages of the minerals in the various pulses. The equivalents in c.c. of normal acid or alkali of the different constituents of the ash are indicated in Table III while Table IV gives the titrable acidity per 100 g. of pulses in c.c. of normal acid,

TABLE II.

Showing the ash constituents as percentage of oxides on moist basis.

Minerals.	Green gram (<i>Phaseolus mungo</i>).	Field pea (<i>Pisum sativum</i>).	Bengal gram (<i>Cicer arietinum</i>).	Lentil (<i>Lens esculenta</i>).	Khesari (<i>Lathyrus sativa</i>).	Soya bean (<i>Glycine hispida</i>).	Arhar (<i>Cajanus indicus</i>).
Silica, SiO_2 ..	0.02	0.0469	0.017	0.0243	0.01416	0.0025	0.03
Potassium oxide, K_2O ..	0.9616	1.118	0.8377	0.7588	0.9396	1.455	1.086
Sodium oxide, Na_2O ..	0.1038	0.1184	0.1017	0.0832	0.1298	0.1749	0.1543
Calcium oxide, CaO ..	0.1491	0.087	0.1474	0.0839	0.0990	0.3141	0.1327
Magnesium oxide, MgO ..	0.1408	0.1666	0.1251	0.0867	0.1526	0.2204	0.1864
Iron oxide, Fe_2O_3 ..	0.0088	0.0064	0.0073	0.0059	0.0101	0.0156	0.0091
Phosphorous oxide, P_2O_5	0.9369	1.046	0.821	0.761	0.9292	1.509	1.131
Sulphur oxide, SO_3 ..	0.4257	0.4739	0.4856	0.3496	0.4534	0.7280	0.5371
Chlorine, Cl ..	0.0314	0.0334	0.0227	0.0336	0.0262	0.0674	0.0389
Total ash, per cent ..	2.81	3.12	3.59	2.2	2.81	4.53	3.28

TABLE III.

Normal alkali in c.c.

Name of pulse.	KOH.	NaOH.	Mg(OH) ₂ .	Ca(OH) ₂ .	Total (c.c. N. alkali).
Green gram ..	20.41	3.31	6.98	5.32	36.02
Field pea ..	23.73	3.81	8.26	3.10	38.90
Bengal gram ..	17.65	3.27	6.20	5.26	32.38
Lentil ..	16.11	2.68	4.30	3.0	26.09
Khesari ..	19.95	4.18	7.56	3.53	35.22
Soya bean ..	30.89	5.64	10.93	11.21	58.67
Arhar ..	23.05	4.9	9.25	4.73	41.93

Normal acid in c.c.

Name of pulse.	Sulphuric acid.	Phosphoric acid.	Hydrochloric acid.	Total (c.c. N. acid).
Green gram ..	26.37	10.64	0.88	37.89
Field pea ..	29.44	11.84	0.94	42.22
Bengal gram ..	23.11	12.14	0.67	35.92
Lentil ..	21.42	8.73	0.95	31.10
Khesari ..	26.15	11.33	0.73	38.21
Soya bean ..	42.48	18.20	1.90	62.58
Arhar ..	31.84	13.42	1.10	46.36

TABLE IV.

*Approximate titrable acidity per 100 grammes
(c.c. normal acid).*

Name of pulse.	Potential acidity.
Green gram (<i>Phaseolus mungo</i>) ..	1.87
Field pea (<i>Pisum sativum</i>) ..	3.32
Bengal gram (<i>Cicer arietinum</i>) ..	3.54
Lentil (<i>Lens esculenta</i>) ..	5.01
Khesari (<i>Lathyrus sativa</i>) ..	3.00
Soya bean (<i>Glycine hispida</i>) ..	3.91
Arhar (<i>Cajanus indicus</i>) ..	4.43

DISCUSSION.

The results recorded in Table IV show that the potential acidities of green gram, field pea, Bengal gram, lentil, khesari, soya bean, and arhar lie between 1.87 and 5.01. Green gram possesses the least acidity (1.87) and this indicates that one who takes green gram in his daily food requires less base-forming foods as fruits and vegetables compared with other pulses to maintain the acid-base balance in the blood and to lower the urinary hydrogen-ion concentration. In the case of lentil the potential acidity has been found to be 5.01 and hence it requires more of the base-forming foods to maintain the balance. Moreover, the results from this laboratory have shown that the biological value of the lentil protein is rather low. Therefore, the popular belief that the lentil is a good variety of pulse from the nutritive point of view is not substantiated.

SUMMARY.

The available carbohydrate in eight varieties of pulses has been determined. It has been found that green gram, field pea, lentil, khesari, and arhar contain nearly all their carbohydrates in the available form. Appreciable amounts of the carbohydrates in soya bean and Bengal gram are in the unavailable form, soya bean carbohydrate giving the lowest value so far as the availability is concerned.

The potential acidities of the above eight pulses have been determined by analysing their ash constituents. The acidity is found to be the least in the case of green gram and highest in the case of lentil. Other pulses possess values in between these two extremes.

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RELATION OF INORGANIC PHOSPHATE TO CARBOHYDRATE METABOLISM IN NORMAL AND DIABETIC SUBJECTS.

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INTRODUCTION.

THE relation of the phosphates, especially the inorganic ones, to carbohydrate metabolism has been of great interest for a long time. Evidences based on experiments, animal and human, put forward by several workers tended to show that there did exist a relationship between the inorganic phosphate and carbohydrate metabolism. It has been suggested that inorganic phosphates played an intermediary part in the utilization of carbohydrate in the system. Fiske (1920, 1921) showed that the injection of glucose caused a temporary diminution in the phosphate output in the urine, followed by a compensatory rise. Blatherwick, Bell and Hill (1924) reported that inorganic phosphates of the blood and urine became diminished after the administration of glucose by mouth. Perlzweig, Lathaham and Keefer (1923) demonstrated the diminution of blood inorganic phosphates after the administration of dextrose and this observation was confirmed by Sokhey and Allan (1924), and Harrop and Benedict (1923, 1924). Bolliger and Hartman (1925) also observed in human subjects the same depression of inorganic phosphate content of blood and urine after the administration of glucose. More recently, Pijoan and Quigley (1937) have shown that intravenous injection of glucose in fasting dogs increased the degree of fall of inorganic phosphates of the blood.

It has also been shown that the injection of insulin caused a marked fall in the plasma inorganic phosphates. This was first demonstrated by Wigglesworth, Woodrow, Smith and Winter (1923) and by Winter and Smith (1924). Similar

results were obtained by Cori and Cori (1933) and Perlzweig, Lathaham and Keefer (*loc. cit.*) and Volmer (1923). They also demonstrated that injection of epinephrin produced the same effect (fall of inorganic phosphates of blood). Briggs, Koechigs, Doisy and Webber (1924) observed that insulin caused the fall of blood inorganic phosphates in dogs. Cori and Cori (1937) found that after the administration of glucose and insulin together the fall of inorganic phosphate content of the muscles took place.

DISTRIBUTION OF PHOSPHATE CONTENT OF NORMAL BLOOD.

According to some observers the inorganic phosphates of the blood are distributed equally between the cells and the plasma; others, however, have put forward evidence to show that the cells contained no measurable amount of inorganic phosphates. Buell (1923) observed that the centrifuged cells of dog's blood were free from inorganic phosphates. In the cells of human beings as little as 0.5 mg. of inorganic phosphates per 100 c.c. of blood was found, but Buell believed that even that was due to the breakdown of the cell compound and that the inorganic phosphate usually found in cells was produced by autolytic hydrolysis during the preliminary treatment of blood. Halpern (1936), however, found that the inorganic phosphate content of the blood was distributed in the serum and the cells, but the concentration in the former was higher than in the latter. Martland, Hansman and Robison (1924) in working out the phosphate content of the laked blood found that if it was kept at room temperature (at 30°C.) for some time the inorganic phosphate content rose considerably. Kay and Robison (1924) corroborated the same results working with unlaked blood.

To find out the distribution of the inorganic phosphates in the blood of human subjects, we started to estimate the phosphate content of the whole blood and the plasma separately in the same sample of blood. After several such estimations, we came to the conclusion that the results obtained from the whole blood were not at all uniform and were likely to vary considerably, particularly if the specimen was kept at room temperature for some time. The results of estimation of the phosphate content of the plasma, however, were found to be more uniform and did not vary much even if kept in room temperature for some time. This was probably due to the fact that in the plasma the acid-soluble phosphates present were entirely inorganic and consisted of such bases as sodium and potassium and as such were not likely to vary under different conditions. The phosphates present in the cells, however, consisted of a mixture of the inorganic and the organic, the proportions of which were not at all constant on account of the interchange between the two continually taking place in accordance with the needs of the organism and thus it tended to produce varying results. The conclusion we arrived at was that in the estimation of the inorganic phosphate content of the blood it was best to leave the cells out and to estimate the phosphate content of the plasma (instead of the whole blood) to get uniformity of results.

USE OF ANTI-COAGULANTS.

The choice of the proper anti-coagulant for the collection of blood for estimation of phosphates appears to have been a subject of controversy for a long time.

Remington (1924) stated that, if the anti-coagulant used exceeded a certain definite proportion, the final development of the blue colour was interfered with, in that it became turbid and hence the exact matching in colour became difficult at times. Gaebler (1932), using sodium fluoride as an anti-coagulant, found it unsuitable but got better results when he used it along with a definite amount of aluminum chloride. Using neutral potassium oxalate, however, Gaebler found that it gave more or less uniform results even if the (whole) blood was left at room temperature for four to five hours.

Burkens (1935) has, however, shown that sodium fluoride was the most suitable as anti-coagulant for the estimation of inorganic phosphate content of blood. In contrast to the observations made by the previous workers, he found that it did not interfere with the development of colour when used in correct amount (10 mg. per 5 c.c. of blood). He, moreover, found that sodium fluoride checked ester-phosphorus hydrolysis and synthesis and hence no variations in results took place even when the blood was kept for hours or days at room temperature or at 30°C.

In our experiments, we tried both neutral potassium oxalate and sodium fluoride separately as anti-coagulants for collection of samples. In two different portions of the same sample of blood we used the above two anti-coagulants separately in the proportion of 2 mg. of either, per c.c. of blood as indicated by Remington and Burken. Simultaneous estimations of plasma inorganic phosphates in two samples were done and practically no difference in the results was observed. No interference with colour development was observed either. Having thus found practically no difference in the results, we preferred to use sodium fluoride as an anti-coagulant, the idea being that it would help the simultaneous estimation of blood-sugar because, as had been stated by previous observers, fluoride stopped both hydrolysis and glycolysis of blood for a considerably long time*.

METHOD OF ESTIMATION.

In our experiments blood was drawn from the vein by means of a dry sterile syringe and 5 c.c. were collected directly into a 15-c.c. graduated centrifuge tube containing 10 mg. of sodium fluoride and was then mixed by rotating between the palms. 0.2 c.c. of blood was immediately measured out for estimation of sugar and the rest was centrifuged and plasma separated. The whole process did not take more than 5 to 10 minutes.

Two c.c. of plasma were then measured out in a pipette and poured drop by drop into a 10-c.c. stoppered cylinder containing 8 c.c. of 10 per cent trichlor-acetic acid with constant shaking. The method of Fiske and Subbarow (1925) which is a modification of the method of Bell and Doisy (1920) was adopted for the estimation of phosphates.

The estimation of blood-sugar was done by the modified Folin and Wu (1919) method.

* Martland and Robison (1924) found that trichlor-acetic acid occasionally contained impurities and produced colour with Bergg's reagent. In order to test the reagents, we tried the method in blank but did not observe any turbidity or development of colour.

INORGANIC PHOSPHATE CONTENT OF BLOOD IN NORMAL HEALTHY INDIVIDUALS.

To ascertain a standard for the inorganic phosphate content of the blood of Indians, we did a series of estimations of blood-phosphates of healthy normal Indian subjects. Estimation of blood-sugar was also done simultaneously; the results are given in Table I :—

TABLE I.

Showing results of estimation of blood-sugar and inorganic phosphate content of plasma of normal Indian subjects.

Serial number.	Date.	Name.	Blood-sugar, per cent.	Blood-phosphate, mg. per cent.
1	12-11-36	P. G.	0.085	3.5
2	12-11-36	M. R.	0.070	3.3
3	23-12-36	S. D.	0.100	3.2
4	23-12-36	M. B.	0.100	3.0
5	4-1-37	A. T.	0.085	3.1
6	4-1-37	M. B.	0.085	3.2
7	5-1-37	B. H.	0.100	3.2
8	6-1-37	J. B.	0.090	2.5
9	7-1-37	D.	0.115	4.0
10	8-1-37	A. G.	0.100	4.0
11	8-1-37	S. B.	0.110	4.6
12	12-1-37	S.	0.065	3.4
13	12-1-37	D.	0.080	3.6
14	21-1-37	A. G.	0.070	3.0
15	21-1-37	M. L.	0.075	3.6
16	19-5-37	J.	0.090	4.2
17	23-3-37	B. S.	0.085	2.3
18	23-3-37	G. D.	0.087	3.6
19	30-3-37	B.	0.080	2.1
20	30-3-37	K.	0.082	3.0
21	30-3-37	S. G.	0.080	3.5
22	2-4-37	R. B.	0.084	3.6

TABLE I—concl'd.

Serial number.	Date.	Name.	Blood-sugar, per cent.	Blood-phosphate, mg. per cent.
23	5-4-37	M. K.	0.070	3.0
24	9-4-37	A. M.	0.074	4.0
25	16-4-37	T. S.	0.100	3.7
26	19-4-37	G. R.	0.103	3.0
27	27-4-37	P. C.	0.080	4.3
28	23-1-37	Mrs. J.	0.105	2.1
29	23-1-37	K. N.	0.083	3.0
30	19-4-37	U. B.	0.105	5.0
31	3-5-37	B.	0.080	3.2
32	7-5-37	A. B.	0.085	3.1
33	7-5-37	P. T.	0.105	3.1
34	8-5-37	M. D.	0.080	3.2
35	10-5-37	O.	0.091	4.6
36	10-5-37	McK.	0.075	3.7
37	11-5-37	C. M.	0.105	3.2
38	12-5-37	Mrs. D.	0.115	4.2
39	15-5-37	A. C.	0.100	4.1
40	19-5-37	H.	0.100	3.4
41	25-3-37	M. D.	0.115	3.0
42	28-8-37	B. S.	0.079	4.1
43	28-8-37	A. R.	0.110	3.2
44	2-6-37	C. G.	0.098	3.5
45	7-6-37	R. A.	0.105	3.8
46	8-6-37	M. H.	0.090	5.0
47	12-6-37	P.	0.092	4.9
48	17-6-37	R. B.	0.100	5.3
49	22-6-37	S. B.	0.089	3.0
50	22-6-37	P. C.	0.090	3.0

An analysis of the result given in Table I brings out the following facts :—

Inorganic phosphate content of the blood, mg. per cent.	Number of cases.	Percentage.
Below 3 ..	4	8
From 3 to 3·9 ..	32	64
„ 4 to 5 .	13	26
Above 5 ..	1	2

It will be evident from Table I that the inorganic phosphate content of normal blood in the majority of cases (32) under investigation was between 3 mg. and 3·9 mg. per cent. The next largest number of cases (13) showed a value between 4 mg. and 5 mg. per cent. Thus, it appears that 90 per cent of the total cases examined showed an inorganic phosphate content of the blood between 3 mg. and 5 mg. per cent. This we consider to be the range of variations in the inorganic phosphate content of blood in normal Indian subjects*.

From a review of the literature on the point it appears that the result obtained by us does not materially differ from the standard European and American figures. It is also evident that race *per se* does not appear to have much effect on the phosphate content of the blood†.

To summarize our results, we find that the *average* inorganic phosphate content of the plasma in healthy normal Indian subjects is 3·5 mg. per 100 c.c., the minimum being 3 mg. per cent and the maximum 5 mg. per cent.

INORGANIC PHOSPHATE CONTENT OF BLOOD OF DIABETIC SUBJECTS.

With a view to find out whether the inorganic phosphate content of the blood in diabetic subjects differed materially from the normal range, systematic investigation of the inorganic phosphate of the blood of diabetic subjects of varying grades of severity was undertaken by us. Blood-sugar estimations were also done simultaneously.

* Only 8 per cent of our total cases gave values below 3 mg. per cent, the lowest limit obtained by us was 2·1 mg. : only 2 per cent of the cases gave values above 5 mg., the maximum limit obtained being 5·3 mg. As these constituted a more or less negligible percentage compared to the total number of cases investigated, we have thought it fit to ignore these figures.

† Similar observation was made by Bose (1934) and also by Bose and De (1936), regarding the sugar and the cholesterol content of the blood.

Table II shows the results obtained:—

TABLE II.

Diabetic cases.

*Showing results of estimation of blood-sugar and inorganic phosphate content of plasma of **diabetic subjects** of varying grades of severity.*

Serial number.	Date.	Name.	Blood-sugar, per cent.	Blood-phosphate, mg. per cent.
1	5-11-36	Miss C.	0.200	4.10
2	5-11-36	Miss G.	0.230	4.20
3	6-1-37	S. K. B.	0.380	4.0
4	6-1-37	A. A.	0.268	3.0
*5	14-1-37	S. K. B.	0.240	6.4
6	4-2-37	M. A.	0.220	3.0
7	8-2-37	K. S.	0.180	3.2
8	18-2-37	Mrs. B.	0.120	3.2
9	18-2-37	Mrs. H.	0.200	3.8
10	18-2-37	Mrs. M.	0.190	2.6
11	20-2-37	K. T.	0.185	3.0
12	18-3-37	A.	0.185	4.2
13	19-3-37	J. M.	0.330	3.8
14	25-3-37	J. R.	0.240	2.7
15	30-3-37	F. H.	0.300	3.1
16	1-4-37	J. R.	0.180	5.0
17	1-4-37	B. B.	0.180	3.9
18	1-4-37	Miss G.	0.198	3.2
19	2-4-37	J.	0.167	4.2

* We would like to point out that only 1 case out of 50 showed an unusual phosphate content of 6.4 mg. per 100 c.c. This was corroborated by a repetition of the test.

TABLE II—*contd.*

Serial number.	Date.	Name.	Blood-sugar, per cent.	Blood-phosphate, mg. per cent.
20	8-4-37	Mrs. B.	0.510	3.6
21	19-4-37	K.	0.740	3.7
22	22-4-37	M. D.	0.175	3.2
23	22-4-37	P. P.	0.200	4.0
24	22-4-37	A. B.	0.198	3.4
25	22-4-37	Mrs. M.	0.175	4.0
26	22-5-37	B. S.	0.390	3.8
27	6-5-37	B.	0.150	4.0
28	6-5-37	A. G.	0.250	4.0
29	13-5-37	B. S.	0.280	3.6
30	13-5-37	B.	0.140	4.1
31	13-5-37	Mrs. B.	0.380	4.1
32	13-5-37	A. G.	0.360	4.1
33	13-5-37	B. S.	0.360	3.3
34	18-5-37	Mrs. B.	0.400	3.6
35	18-5-37	B. S.	0.275	3.0
36	18-5-37	A. G.	0.360	4.0
37	18-5-37	B.	0.135	3.9
38	3-6-37	N. S.	0.170	3.2
39	4-6-37	S.	0.250	3.7
40	5-6-37	S.	0.190	4.3
41	14-6-37	A. M.	0.151	3.8
42	15-6-37	Mrs. E.	0.341	3.3
43	16-6-37	P. A.	0.326	3.7
44	16-6-37	P. S.	0.205	3.5
45	16-6-37	S. G.	0.400	4.2
46	19-6-37	S. P.	0.195	2.8

TABLE II—*concl.*

Serial number.	Date.	Name.	Blood-sugar, per cent.	Blood-phosphate, mg. per cent.
47	19-6-37	M. A.	0.200	4.3
48	21-6-37	Mrs. B.	0.360	3.8
49	21-6-37	C. R.	0.220	4.2
50	21-6-37	R. M.	0.189	3.7

An analysis of the results given in Table II brings out the following facts :—

Inorganic phosphate content of the blood, mg. per cent.	Number of cases.	Percentage.
Below 3 ..	3	6
From 3 to 5 ..	46	92
Above 5 ..	1	2

A careful analysis of Table II shows that out of 50 cases of diabetes examined, 46 (92 per cent) cases showed a normal inorganic phosphate content. Out of the remaining 4 cases, 3 cases (6 per cent) showed a slightly lower phosphate content than the accepted normal.

It thus becomes evident that under normal 'fasting' conditions, the phosphate content of blood in diabetic subjects remains more or less within normal limits though the blood-sugar in some of the cases is extremely high. The blood-sugar thus appears to bear no relationship to the phosphate content of the blood under normal 'fasting' conditions.

EFFECT OF INGESTION OF GLUCOSE ON THE PHOSPHATE CONTENT OF THE BLOOD IN NORMAL INDIVIDUALS.

As stated before, there is a good deal of evidence in the literature to show that the blood-phosphates play an important part during the stage of active carbohydrate metabolism.

With a view to studying in what way the inorganic phosphates help the carbohydrate metabolism, we planned the following line of investigation :—

We selected 20 healthy normal individuals and after giving them a load of 100 grammes of glucose by mouth, carried out simultaneous estimations of blood-sugar and blood-phosphates before administration of glucose and then every half hour after, for a period of two hours.

Table III shows the results obtained :—

TABLE III.

Showing the effect of oral administration of glucose on the sugar and the inorganic phosphate content of the blood in normal healthy subjects.

Serial number.	Name.	BLOOD-SUGAR, MG. PER 100 C.C.					BLOOD-PHOSPHATE, MG. PER 100 C.C.				
		Before.	$\frac{1}{2}$ hour after.	1 hour after.	$1\frac{1}{2}$ hours after.	2 hours after.	Before.	$\frac{1}{2}$ hour after.	1 hour after.	$1\frac{1}{2}$ hours after.	2 hours after.
1	O. ..	91	140	106	100	90	4.6	4.0	3.2	3.7	4.4
2	M. ..	75	141	105	95	91	3.7	3.2	2.7	2.9	3.3
3	C. M. ..	105	180	105	100	92	3.2	2.4	2.2	2.6	3.2
4	D. ..	105	190	110	98	100	4.2	3.6	3.3	3.5	4.4
5	H. ..	95	170	115	100	100	3.4	2.5	2.3	2.5	3.2
6	D. D. ..	95	145	110	90	95	3.2	2.6	2.4	2.8	3.3
7	D. ..	103	175	110	96	90	3.0	2.6	2.3	2.5	2.9
8	A. ..	97	160	95	90	108	3.2	2.6	2.3	2.6	3.3
9	K. B. ..	100	158	115	98	93	3.2	2.7	2.2	2.6	2.8
10	B. T. ..	103	159	106	90	90	3.1	2.6	2.4	2.7	3.0
11	C. L. B.	90	160	110	89	88	3.2	2.5	2.2	2.8	3.0
12	A. D. C.	100	175	114	100	100	4.0	3.4	3.1	3.4	3.7
13	C. R. ..	102	198	108	100	98	3.7	3.0	2.5	2.9	3.8
14	N. M. ..	85	180	104	92	102	3.0	2.5	2.0	2.2	2.8
15	A. B. ..	79	190	115	95	100	3.1	2.5	2.2	2.5	2.8
16	L. M. ..	100	165	110	90	90	4.1	3.3	3.0	3.4	3.8
17	K. N. M.	98	156	100	97	100	3.9	3.1	3.0	2.7	3.7
18	A. H. ..	105	168	100	90	92	4.5	3.8	3.4	3.6	4.5
19	J. N. ..	90	175	119	90	87	3.0	2.4	2.1	2.5	2.8
20	B. N. M.	100	175	112	100	100	3.4	2.5	2.2	2.7	3.2
AVERAGE		95	168	108	95	94	3.53	2.89	2.50	2.85	3.39

SUMMARY OF THE EFFECT OF INGESTION OF 100 GRAMMES OF GLUCOSE ON
THE SUGAR AND THE PHOSPHATE CONTENT OF BLOOD IN
NORMAL INDIVIDUALS.

Effect on blood-sugar.—It will be seen from Table III that :—

- (1) The blood-sugar begins to rise after the ingestion of glucose, the maximum rise taking place usually half an hour after the glucose meal.
- (2) The blood-sugar, thereafter, begins to fall sharply and comes down to near about the original level, usually within one to one and a half hours.

Effect on blood-phosphates.—The phosphate content of the blood, however, behaves differently as follows :—

- (1) It begins to fall quickly after the ingestion of glucose so that a considerable decrease in the phosphate content of the blood takes place half an hour after the glucose meal. The maximum decrease in the blood-phosphates, however, usually takes place one hour after the glucose meal.
- (2) The blood-phosphates thereafter begin to rise and usually reach the original (pre-glucose) level two hours after the glucose meal.

It will thus be seen that the blood-sugar and the blood-phosphate curves, after the test-meal glucose, take almost reverse directions, i.e., during the period of rise of blood-sugar after the glucose meal and also until the blood-sugar comes back to normal (or nearly normal) level, *the phosphate content of the blood decreases continuously.*

Thereafter, the blood-phosphates begin to rise, even though the blood-sugar level either continues to fall still further or remains at the same level.

We thus see that during the process of active carbohydrate metabolism in the system, the phosphate content of the blood falls continuously. As soon as the blood-sugar, however, comes back to the normal level, the decrease in the phosphate content stops and it slowly begins to rise again.

It seems to be clear from the above experiment that the blood-phosphates play an important part in the metabolism of carbohydrates in the system. What appears to happen following the ingestion of carbohydrates may be stated as follows : that a portion of the inorganic phosphate content of the blood is released, which combines with the excess of the glucose in blood and forms a compound, probably hexose-phosphates, in which 'intermediary' form the blood-glucose is known to be utilized in the system. The process thus accounts for the sharp fall in the phosphate content of the plasma during the first half hour after the glucose meal. The fall in the inorganic phosphate content of the blood, however, continues, though somewhat slowly, during the next half hour, i.e., till the blood-sugar level comes back to normal. As soon as the blood-sugar comes down to the normal or nearly normal level the demand for the inorganic phosphates for the active utilization of glucose in the system ceases and thus the steady fall of blood-phosphates is discontinued. It will, thus, appear that during the stage of active carbohydrate metabolism there is a steady and continuous fall in the plasma phosphates, which,

however, appears to be of temporary nature and lasts as long as the active glucose utilization takes place in the system, at the end of which period the phosphate value of the blood again returns to the initial level.

EFFECT OF INGESTION OF GLUCOSE ON THE PHOSPHATE CONTENT OF
BLOOD IN DIABETIC SUBJECTS.

Having got such interesting data in the above experiments, namely, the important part which the phosphates play in the utilization of carbohydrates in the system in normal subjects, we proceeded to conduct similar experiments on diabetic subjects. With this object in view we selected cases from the mild, moderate and severe types of diabetes; the results are given in Table IV:—

TABLE IV.

Showing the effect of oral administration of glucose on the sugar and the phosphate content of the blood in diabetic subjects.

Serial number.	Name.	BLOOD-SUGAR, MG. PER 100 C.C.					BLOOD-PHOSPHATE, MG. PER 100 C.C.				
		Before.	$\frac{1}{2}$ hour after.	1 hour after.	$1\frac{1}{2}$ hours after.	2 hours after.	Before.	$\frac{1}{2}$ hour after.	1 hour after.	$1\frac{1}{2}$ hours after.	2 hours after.
<i>Group A—mild cases of diabetes.</i>											
1	P. G. ..	112	225	205	168	158	4·8	4·2	4·0	3·9	3·8
2	A. T. M.	114	220	180	158	154	3·7	3·0	2·8	2·8	3·0
3	B. C. G.	115	240	178	160	144	4·3	3·8	3·6	3·4	3·6
4	P. K. ..	110	252	180	150	138	5·0	4·5	4·3	4·1	4·3
5	B. C. ..	113	212	200	170	159	3·7	3·1	2·9	2·7	3·0
6	R. D. ..	106	243	190	178	160	5·2	4·7	4·3	4·0	4·4
AVERAGE ..		111	232	189	162	151	4·4	3·8	3·6	3·4	3·6

TABLE IV—concl'd.

Serial number.	Name.	BLOOD-SUGAR, MG. PER 100 C.C.					BLOOD-PHOSPHATE, MG. PER 100 C.C.				
		Before.	$\frac{1}{2}$ hour after.	1 hour after.	$1\frac{1}{2}$ hours after.	2 hours after.	Before.	$\frac{1}{2}$ hour after.	1 hour after.	$1\frac{1}{2}$ hours after.	2 hours after.
<i>Group B—moderate cases of diabetes.</i>											
1	S. K. G.	125	200	245	200	180	3.7	3.5	3.3	3.2	3.0
2	U. N. D.	160	200	270	250	210	4.2	4.0	3.8	3.5	3.4
3	H. K. B.	130	250	300	280	170	3.9	3.7	3.5	3.3	3.2
4	P. K. P.	140	240	260	240	200	5.4	5.0	4.8	4.7	4.7
5	J. B. S.	120	250	330	296	188	4.5	4.4	4.1	4.1	3.9
6	P. K. B.	135	187	200	185	172	5.0	4.8	4.7	4.6	4.5
7	B. N. D.	152	228	242	225	180	5.4	5.2	5.0	4.8	4.5
AVERAGE ..		131	222	263	239	185	4.4	4.3	4.1	4.0	3.8

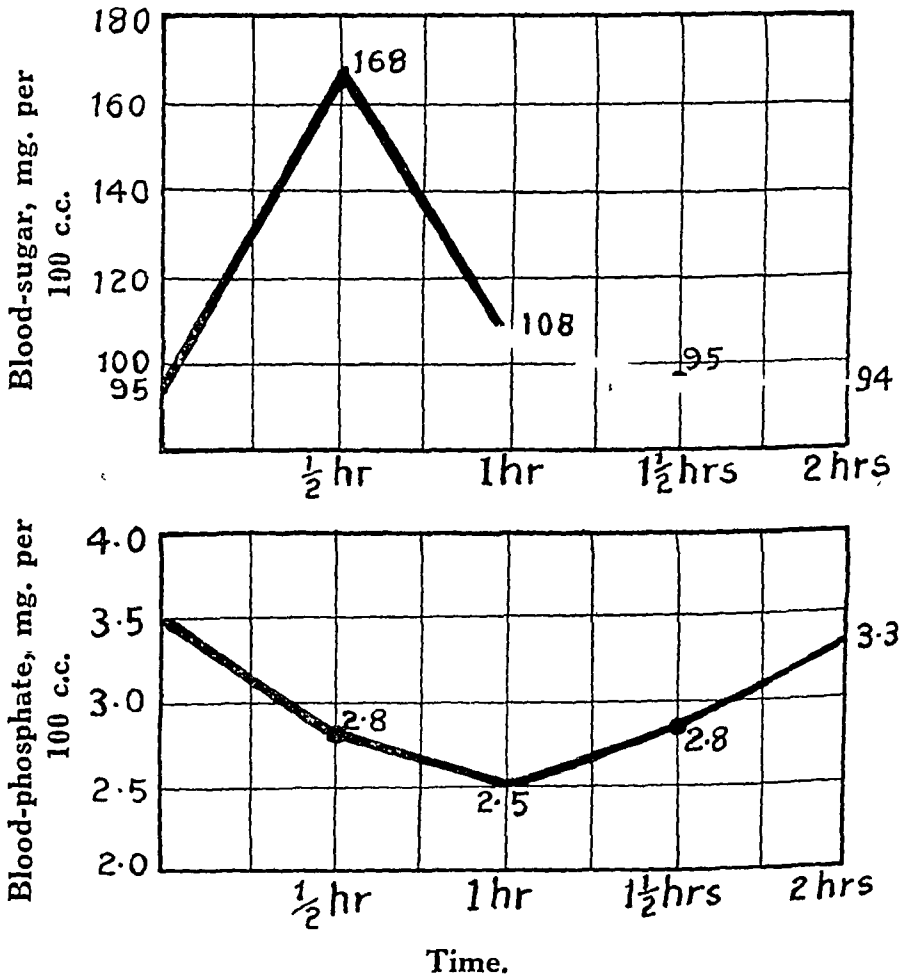
Group C—severe cases of diabetes.

1	L. T. M.	250	362	390	400	400	3.7	3.7	3.5	3.5	3.4
2	F. N. ..	235	340	374	386	370	4.3	4.3	4.3	3.8	3.8
3	S. L. P.	314	380	304	526	552	3.9	3.9	3.8	4.0	4.0
4	D. N. M.	297	423	598	600	585	4.7	4.9	5.0	5.0	5.0
5	H. L. S.	326	418	480	620	540	3.7	3.5	3.5	3.6	3.6
6	B. N. ..	205	342	400	454	446	3.5	3.5	3.4	3.4	3.1
7	S. P. ..	200	305	390	350	320	5.2	5.2	5.4	5.4	5.3
8	P. K. M.	300	420	520	570	550	2.7	2.7	2.8	2.6	2.5
AVERAGE ..		273	373	454	484	470	3.9	3.9	3.9	3.9	3.8

From an analysis of the results obtained in Table IV it is evident that, in diabetic subjects, after the ingestion of glucose, variations in the inorganic phosphate content of blood take place, according to whether the case is mild, moderate or severe. It is also clearly evident that these variations in the results are markedly different from those observed in normal cases (cf. Table III).

In order to summarize the results obtained by us, viz., the effect of oral administration of glucose on the sugar and phosphate content of blood both

GRAPH 1.

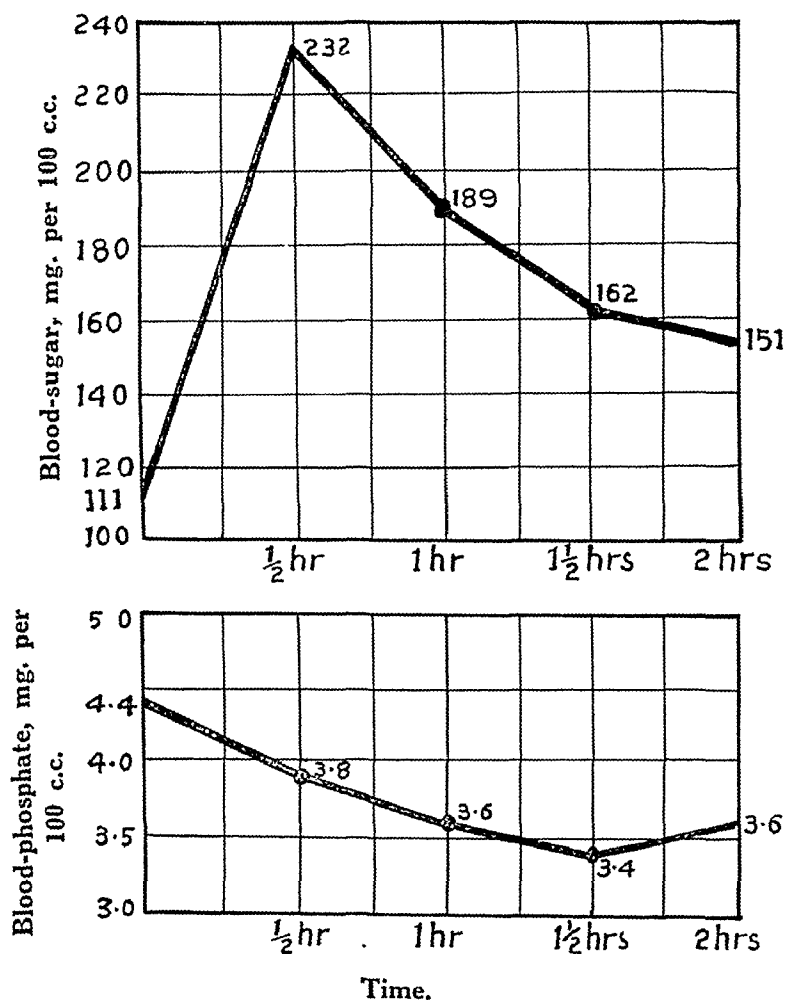


The average blood-sugar and the blood-phosphate curve in healthy normal individuals after ingestion of glucose.

in normal and diabetic subjects, we calculated the *average* values obtained in each group of cases separately and represented them graphically in Graphs 1, 2, 3,

and 4. This gives a clear exposition of the results we have obtained and at the same time makes it easily comparable.

GRAPH 2.

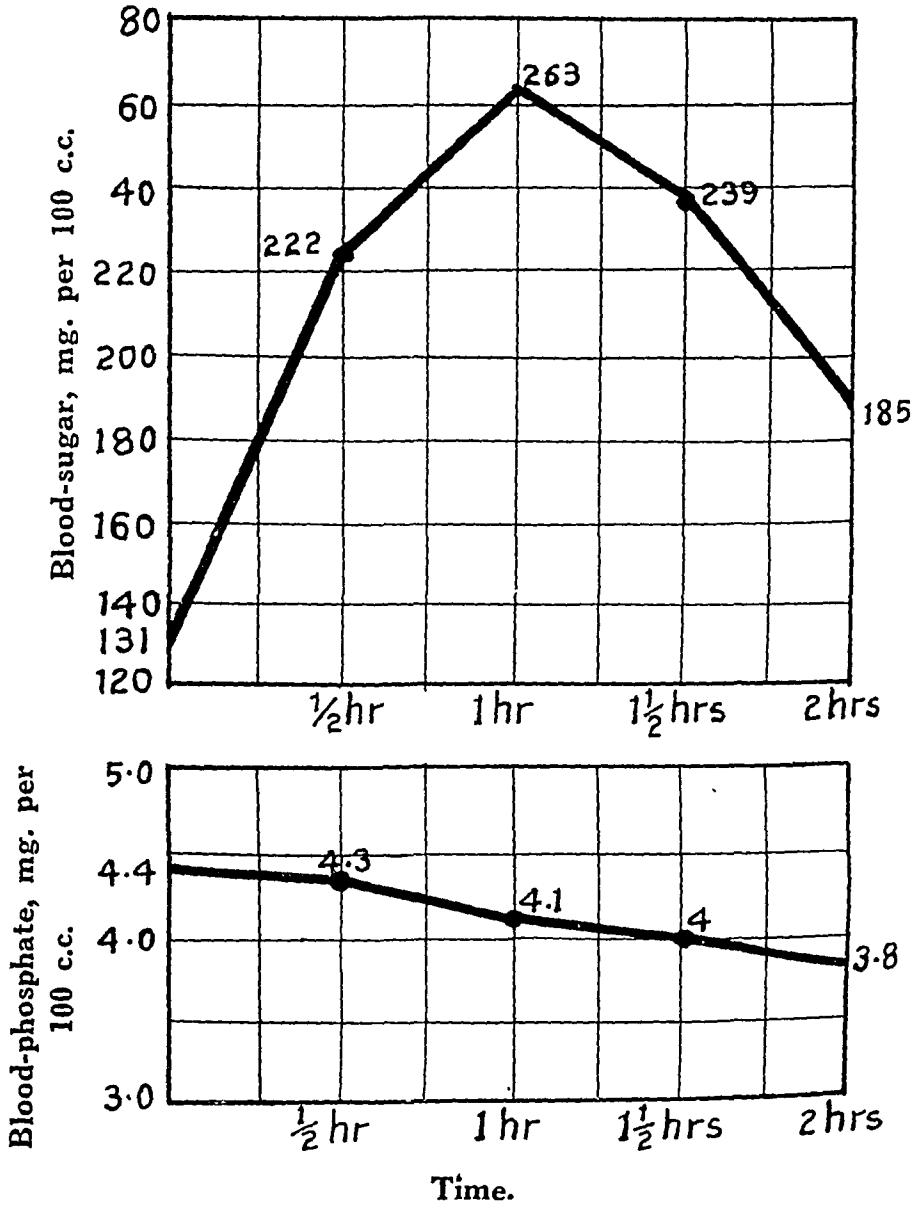


The average blood-sugar and blood-phosphate curve in mild diabetes after ingestion of glucose.

From a careful study of the data obtained by us so far, it thus becomes quite evident that the decrease in the phosphate content of the blood after a glucose meal gives an indication of utilization of sugar in the system.

To make an actual comparative study as to the quantitative decrease in the phosphate content of the blood, at different intervals, after a glucose meal, of

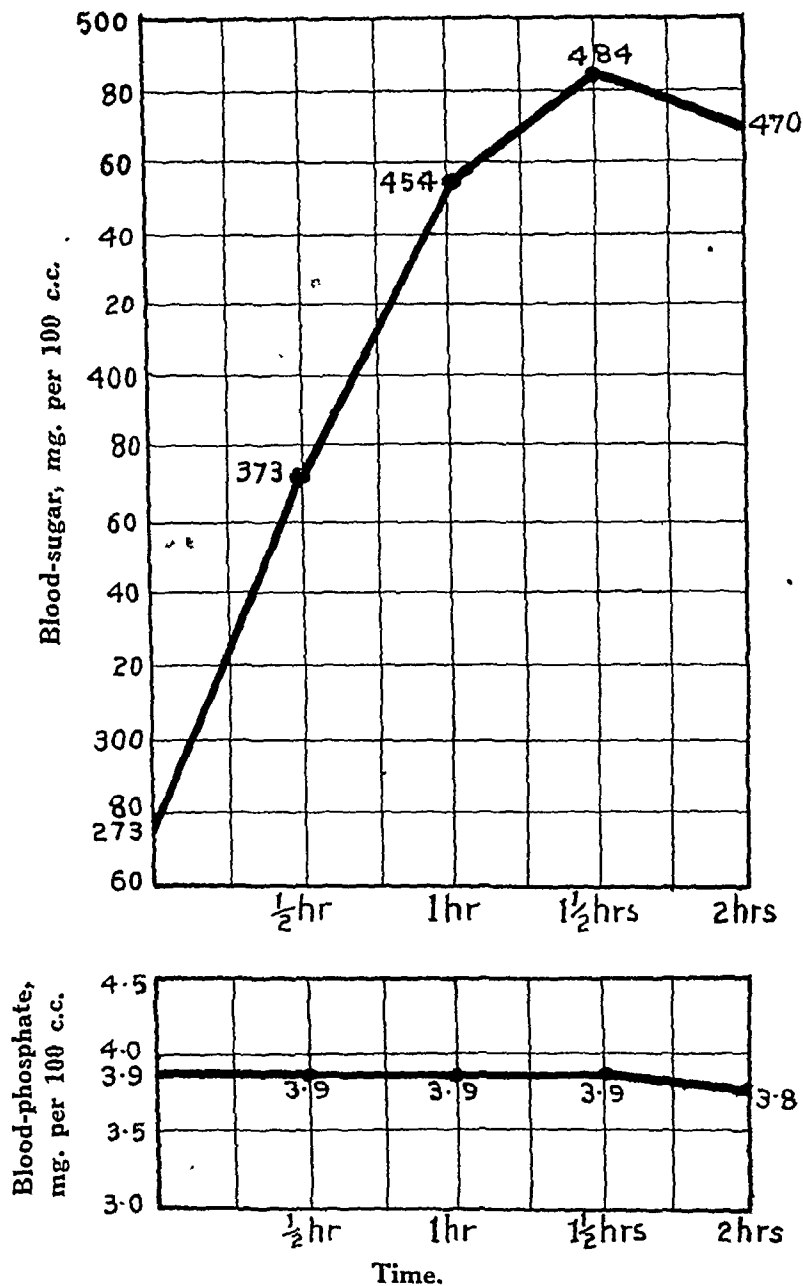
GRAPH 3.



The average blood-sugar and the blood-phosphate curve in moderate diabetes after ingestion of glucose.

normal and diabetic subjects, we are appending below a table (Table V) showing the quantitative decrease in the phosphate content of the blood (from the initial

GRAPH 4.



The average blood-sugar and the blood-phosphate curve in severe diabetes after ingestion of glucose.

'fasting' level) at the half-hourly periods after glucose meal, for a period of 2 hours:—

TABLE V.

Showing the half-hourly quantitative decrease in the phosphate value of the blood (from the initial level) after glucose meal.

Serial number.	Decrease $\frac{1}{2}$ hour after glucose.	Decrease 1 hour after glucose.	Decrease $1\frac{1}{2}$ hours after glucose.	Decrease 2 hours after glucose.
<i>Normal healthy individuals.</i>				
1	0.6	1.4	0.9	0.2
2	0.5	1.0	0.8	0.4
3	0.8	1.0	0.6	0.0
4	0.6	0.9	0.7	0.2
5	0.9	1.1	0.9	0.2
6	0.6	0.8	0.4	0.1
7	0.4	0.7	0.5	0.1
8	0.6	0.9	0.6	0.1
9	0.5	1.0	0.6	0.4
10	0.5	0.7	0.4	0.1
11	0.7	1.0	0.4	0.2
12	0.6	0.9	0.6	0.3
13	0.7	1.2	0.8	0.1
14	0.5	1.0	0.8	0.2
15	0.6	0.9	0.6	0.3
16	0.8	1.1	0.7	0.3
17	0.8	0.9	1.2	0.2
18	0.7	1.1	0.9	0.0
19	0.6	0.9	0.5	0.2
20	0.9	1.2	0.7	0.2
AVERAGE DECREASE.	0.64	0.98	0.68	0.19

TABLE V—*contd.*

Serial number.	Decrease $\frac{1}{2}$ hour after glucose.	Decrease 1 hour after glucose.	Decrease $1\frac{1}{2}$ hours after glucose.	Decrease 2 hours after glucose.
----------------	--	--------------------------------	--	---------------------------------

Mild cases of diabetes.

1	0.6	0.8	0.9	1.0
2	0.7	0.9	0.9	0.7
3	0.5	0.7	0.9	0.7
4	0.5	0.7	0.9	0.7
5	0.6	0.8	1.0	0.7
6	0.5	0.9	1.2	0.8
AVERAGE DECREASE.	0.56	0.80	0.96	0.76

Moderate cases of diabetes.

1	0.2	0.4	0.5	0.7
2	0.2	0.4	0.7	0.8
3	0.2	0.5	0.6	0.7
4	0.4	0.6	0.7	0.7
5	0.1	0.4	0.4	0.6
6	0.2	0.3	0.4	0.5
7	0.2	0.4	0.6	0.9
AVERAGE DECREASE.	0.2	0.4	0.5	0.7

TABLE V—*concl.*

Serial number.	Decrease $\frac{1}{2}$ hour after glucose.	Decrease 1 hour after glucose.	Decrease $1\frac{1}{2}$ hours after glucose.	Decrease 2 hours after glucose.
<i>Severe cases of diabetes.</i>				
1	0.0	0.2	0.2	0.3
2	0.0	0.0	0.5	0.5
3	0.0	0.1	+0.1	+0.1
4	+0.2	+0.3	+0.3	+0.3
5	-0.2	0.2	0.1	0.1
6	0.0	0.1	0.1	0.4
7	0.0	+0.2	+0.2	+0.1
8	0.0	+0.1	0.1	0.2
AVERAGE DECREASE.	0.0	0.0	0.05	0.12

From an analysis of the results given in Table V we find:—

(1) That in normal healthy individuals, the *average* decrease in the blood-phosphates (from the *initial* level) is as follows:—

- (A) 0.64 mg. at the end of the $\frac{1}{2}$ -hour period.
 (B) 0.98 " " " " " " " 1 " "
 (C) 0.86 " " " " " " " $1\frac{1}{2}$ " "
 (D) 0.19 " " " " " " " 2 " "

(2) That in mild cases of diabetes the *average* decrease in the blood-phosphates (from the *initial* level) is as follows:—

- (A) 0.56 mg. at the end of the $\frac{1}{2}$ -hour period.
 (B) 0.80 " " " " " " " 1 " "
 (C) 0.96 " " " " " " " $1\frac{1}{2}$ " "
 (D) 0.76 " " " " " " " 2 " "

(3) That in moderately severe cases of diabetes the *average* decrease in the blood-phosphates (from the *initial* level) is as follows:—

- (A) 0.2 mg. at the end of the $\frac{1}{2}$ -hour period.
 (B) 0.4 " " " " " " " 1 " "
 (C) 0.5 " " " " " " " $1\frac{1}{2}$ " "
 (D) 0.7 " " " " " " " 2 " "

(4) That in the severe cases of diabetes the *average* decrease in the blood-phosphates (from the *initial* level) is as follows:—

- (A) 0.0 mg. at the end of the $\frac{1}{2}$ -hour period.
 (B) 0.0 " " " " " " " 1 " "
 (C) 0.05 " " " " " " " $1\frac{1}{2}$ " "
 (D) 0.12 " " " " " " " 2 " "

From an analysis of the above results we also find that, in normal healthy individuals, the blood-phosphates, after the glucose meal, go on decreasing up to the end of the 1-hour period, i.e., till the blood-sugar comes down to normal or nearly normal level (Graph 1). After this period, however, no further decrease of the phosphate content takes place; the phosphate content in fact begins to rise and this continues till the end of the 2-hour period, when the blood-phosphate almost attains the original level.

In mild cases of diabetes, the results are only slightly different from normal. Here we find that the behaviour of the blood-phosphates, as regards the quantitative rate of fall, is somewhat slower and continues till the end of the $1\frac{1}{2}$ -hour period, as against a definite rise, during the same period, in normal cases. It is only during the 4th half-hour period that the phosphate content of the blood begins to rise slowly, though it is still much behind the initial level at the end of 2 hours after the glucose meal. It thus appears that, in this group of cases, the rate of utilization of sugar is slower and takes a longer time than normal.

In the moderately severe cases of diabetes, the results differ from the mild cases in that the quantitative rate of fall of the phosphate content of blood, after the glucose meal, is slower and continues throughout the entire period of observation, showing thereby that the rate of utilization of sugar is very poor.

In the case of severe diabetes, we get striking results. There is practically no decrease in the phosphate content of the blood after the test meal of glucose indicating thereby that there is little or no attempt at utilization of sugar in the system*.

It can thus be assumed that the lack of utilization of sugar in the system is reflected directly on the changes in the inorganic phosphate content of the blood after a test meal of glucose. We have seen that in the severe types of diabetes, the decrease in the blood-phosphates is practically nil even two hours after the test-meal of glucose, showing thereby that there is no attempt on the part of the system to utilize glucose.

EFFECT OF INJECTION OF INSULIN ON THE PHOSPHATE CONTENT OF BLOOD IN NORMAL AND DIABETIC SUBJECTS.

In order to study as to what happens when insulin is administered in such cases, we selected a few cases of clinically severe diabetes and proceeded to study the effect of insulin on the inorganic phosphate content of the blood, simultaneously with blood-sugar estimation.

To enable us to compare our results, we extended similar studies on normal subjects as well as on mild cases of diabetes. The dose of insulin in each group of cases was kept constant so as to get comparable results.

Table VI shows the effect of 20 units of insulin on the simultaneous sugar and inorganic phosphate content of the blood in each of the three groups of cases, viz., normal, mild and severe diabetes.

* In a few of these cases, however, there was slight rise of blood-phosphate value over the initial level instead of a fall. The reason for this is not clearly understood.

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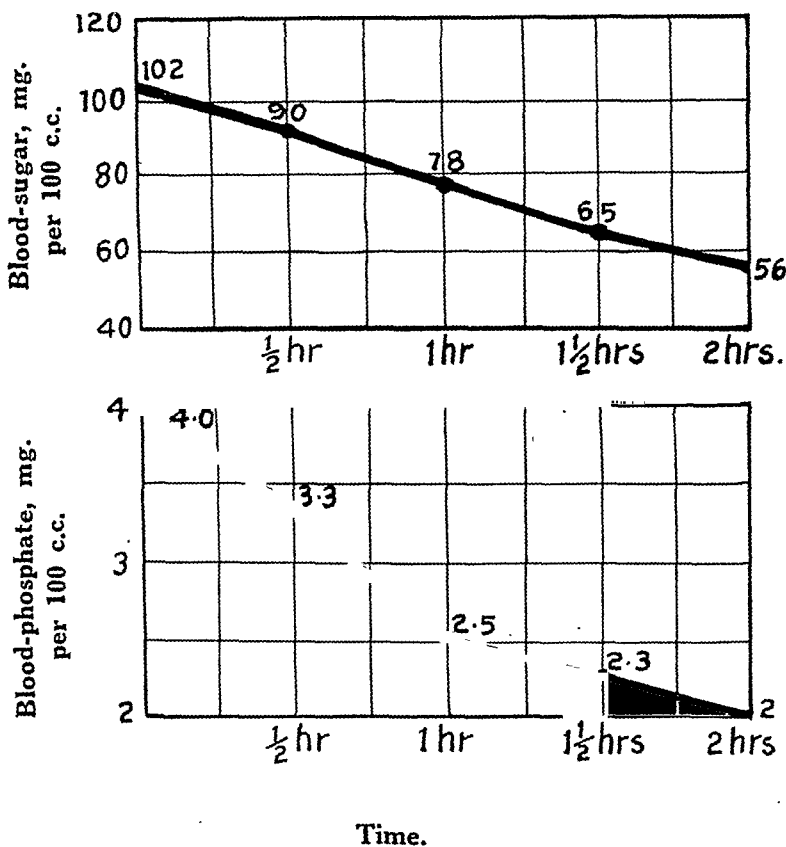
TABLE VI.

Showing the effect of insulin on the sugar and the inorganic phosphate content of blood in normal individuals and in mild and severe diabetes.

Serial number.	Name.	BLOOD-SUGAR, MG. PER 100 C.C.					BLOOD-PHOSPHATE, MG. PER 100 C.C.				
		Before.	$\frac{1}{2}$ hour after.	1 hour after.	1 $\frac{1}{2}$ hours after.	2 hours after.	Before.	$\frac{1}{2}$ hour after.	1 hour after.	1 $\frac{1}{2}$ hours after.	2 hours after.
Normal healthy individuals.											
1	B. C. ..	100	90	80	60	55	4.7	3.6	3.0	2.8	2.6
2	M. N. ..	103	86	77	61	54	3.9	3.0	2.6	2.0	1.9
3	L. M. ..	100	95	80	63	58	3.4	3.0	2.4	1.8	1.6
4	D. C. ..	108	96	88	73	60	4.3	3.6	3.0	2.5	2.2
5	P. C. ..	105	84	72	60	53	4.0	3.5	2.7	2.3	2.0
6	P. N. ..	101	92	80	74	61	3.8	3.2	2.8	2.4	2.0
AVERAGE		102	90	78	65	56	4.0	3.3	2.5	2.3	2.0
Mild cases of diabetes.											
1	B. M. ..	160	117	100	86	78	3.8	3.6	3.2	2.8	2.0
2	T. M. ..	115	100	90	83	70	4.2	3.6	3.0	2.8	2.3
3	D. C. ..	123	100	80	70	68	3.6	3.0	2.6	2.2	1.9
4	S. K. ..	222	194	150	130	125	4.4	3.6	3.1	2.8	2.5
5	H. C. ..	130	150	85	70	63	5.1	4.2	3.7	3.4	3.0
AVERAGE		150	123	101	87	80	4.2	3.6	3.1	2.8	2.3
Severe cases of diabetes.											
1	P. K. ..	328	290	280	274	270	3.5	3.0	3.0	3.2	3.5
2	K. L. ..	300	260	240	236	235	4.7	4.2	4.0	4.5	4.6
3	T. M. ..	312	270	240	232	232	3.9	3.2	2.8	3.6	3.6
4	U. N. ..	396	360	342	234	334	4.5	3.9	3.7	4.0	4.3
AVERAGE		334	295	275	269	267	4.1	3.5	3.3	3.7	4.0

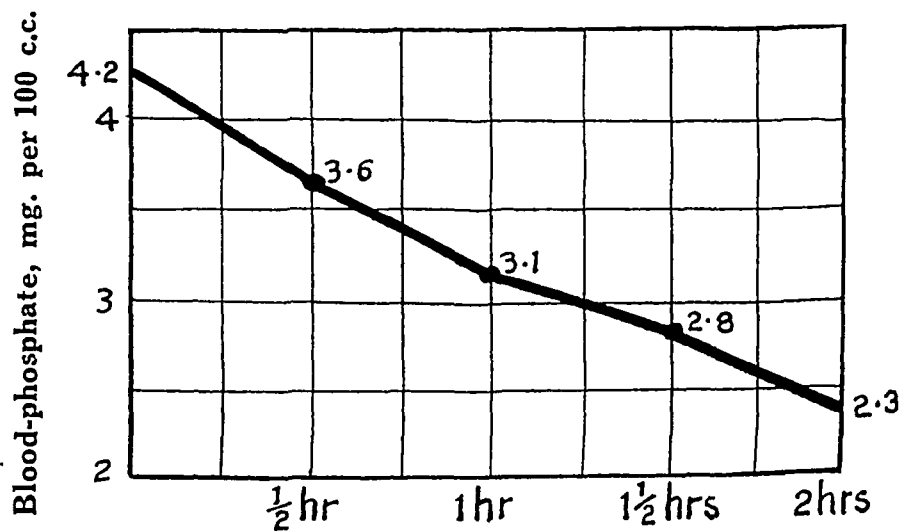
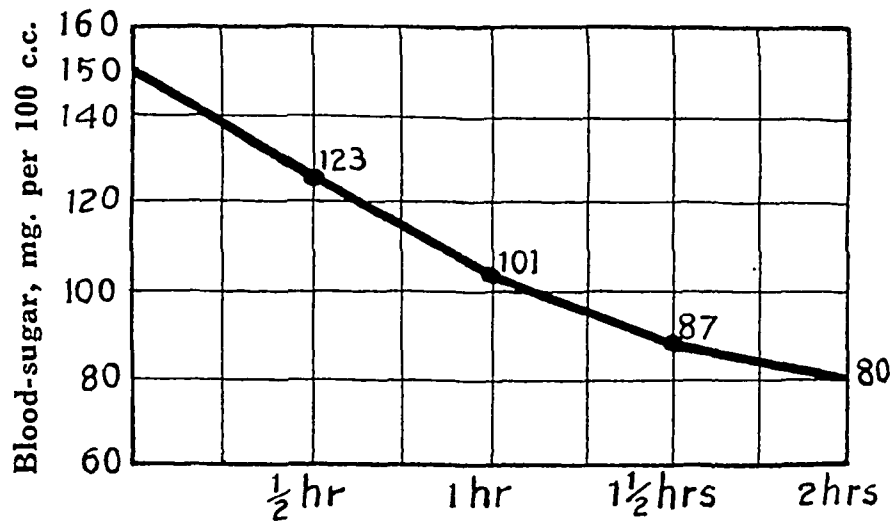
In order to give a clear exposition of the above results, we have represented the *average* results obtained in each of the above group of cases graphically as follows:—

GRAPH 5.



The average blood-sugar and the blood-phosphate curve in healthy normal individuals after a dose of 20 units of insulin.

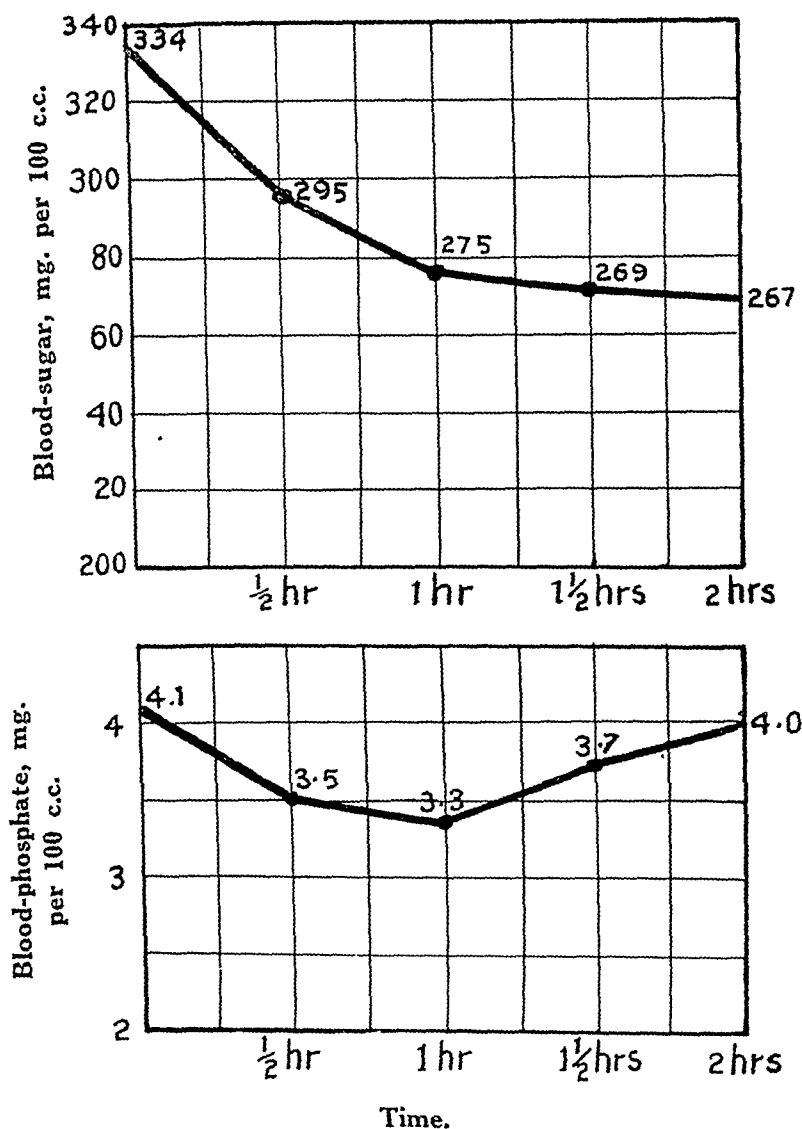
GRAPH 6.



Time.

The average blood-sugar and the blood-phosphate curve in mild diabetes after a dose of 20 units of insulin.

GRAPH 7.



The average blood-sugar and blood-phosphate curve in severe diabetes after a dose of 20 units of insulin.

To make an actual comparative study as to the quantitative decrease in the phosphate content of the blood after insulin injection in normal and diabetic subjects, we are appending below a table (Table VII) showing the quantitative

decrease in the phosphate content of the blood from the initial 'fasting' level at half-hourly periods after insulin for a period of two hours :—

TABLE VII.

Showing the half-hourly quantitative decrease in the phosphate value of the blood (from the initial level) after insulin injection.

Serial number.	Decrease $\frac{1}{2}$ hour after insulin.	Decrease 1 hour after insulin.	Decrease $1\frac{1}{2}$ hours after insulin.	Decrease 2 hours after insulin.
----------------	--	--------------------------------	--	---------------------------------

Normal healthy individuals.

1	0.7	1.7	1.9	2.1
2	0.9	1.3	1.9	2.0
3	0.4	1.0	1.6	1.8
4	0.7	1.3	1.8	2.1
5	0.5	1.3	1.7	2.0
6	0.6	1.0	1.4	1.8
AVERAGE DECREASE.	0.63	1.26	1.70	1.96

Mild cases of diabetes.

1	0.2	0.6	1.0	1.8
2	0.6	1.2	1.4	1.9
3	0.6	1.0	1.4	1.7
4	0.8	1.3	1.6	1.9
5	0.9	1.4	1.7	2.1
AVERAGE DECREASE.	0.62	1.10	1.42	1.88

TABLE VII—concl'd.

Serial number.	Decrease $\frac{1}{2}$ hour after insulin.	Decrease 1 hour after insulin.	Decrease $1\frac{1}{2}$ hours after insulin.	Decrease 2 hours after insulin.
<i>Severe cases of diabetes.</i>				
1	0.5	0.5	0.3	0.0
2	0.5	0.4	0.2	0.1
3	0.7	1.1	0.3	0.1
4	0.6	0.2	0.0	0.0
AVERAGE DECREASE.	0.57	0.55	0.25	0.10

From an analysis of the results obtained in Table VII we find :—

(1) That in normal healthy individuals the *average* decrease in blood-phosphates from the *initial* 'fasting' level is as follows :—

- (A) 0.63 mg. at the end of the $\frac{1}{2}$ -hour period.
 (B) 1.26 " " " " " " " 1 " "
 (C) 1.70 " " " " " " " $1\frac{1}{2}$ " "
 (D) 1.96 " " " " " " " 2 " "

(2) That in mild cases of diabetes the *average* decrease in the blood-phosphates from the initial level is as follows :—

- (A) 0.62 mg. at the end of the $\frac{1}{2}$ -hour period.
 (B) 1.10 " " " " " " " 1 " "
 (C) 1.42 " " " " " " " $1\frac{1}{2}$ " "
 (D) 1.88 " " " " " " " 2 " "

(3) That in the severe cases of diabetes, the *average* decrease in the blood-phosphates from the initial level is as follows :—

- (A) 0.57 mg. at the end of the $\frac{1}{2}$ -hour period.
 (B) 0.55 " " " " " " " 1 " "
 (C) 0.25 " " " " " " " $1\frac{1}{2}$ " "
 (D) 0.10 " " " " " " " 2 " "

From an analysis of the results obtained in the above groups of cases we find that :—

- (1) In normal healthy individuals, a fair degree of fall in the blood-phosphate content starts almost immediately after the insulin injection and this is continued simultaneously with a marked fall in the blood-sugar content. This clearly indicates that active glucose utilization

continues throughout the period of observations under the influence of insulin.

- (2) In mild cases of diabetes, the fall of blood-phosphates also starts immediately after insulin injection and the rate of fall is also similar to that of normal.
- (3) In severe cases of diabetes, though there is a fall of blood-phosphates after insulin injection, the rate of fall is definitely slower, as compared to the normal. The fall in the blood-phosphates continues steadily for about an hour and then it slowly begins to rise. The behaviour of the blood-sugar also is strikingly similar, i.e., there is a sharp fall of blood-sugar for about an hour after the insulin injection and the rate of fall thereafter is definitely slow.

The above results lead us to important and interesting conclusions. It definitely shows that the mild cases of diabetes behave exactly like normal under the influence of insulin as regards the utilization of sugar in the tissues. The severe cases of diabetes also show a very marked improvement in the sugar utilization power under the influence of insulin, though the action is transient and variable.

DISCUSSION.

The results which we have so far obtained clearly indicates that there exists a close relationship between the carbohydrate metabolism and inorganic phosphates of the blood. The phosphate is found to be decreased after ingestion of glucose and also after injection of insulin.

It, therefore, appears that a demand for the inorganic phosphates is created as a result of either glucose ingestion or insulin injection, in normal and diabetic subjects as well, but the degree of the demand appears to vary. We have seen that, in normal subjects, the decrease in the phosphate content of the blood is much more pronounced after insulin injection as regards the depth of the fall and its duration than after glucose ingestion.

Similar kind of result is also observed in the case of diabetic subjects though there is a good deal of difference in the degree, depending on whether the case is mild, moderate or severe.

The important question that naturally arises is—what is responsible for the decrease of the phosphate content of the blood after the ingestion of glucose? It seems reasonable to think that insulin plays an important part in the mechanism for obvious reasons. We know that when glucose is ingested it is absorbed into the portal capillaries and conveyed to the liver where it is partly transformed into glycogen, and partly passed out into the systemic circulation producing temporary hyperglycæmia. The hyperglycæmia thus produced stimulates the pancreas to secrete the necessary extra amount of insulin which ultimately is responsible for the removal of the excess of sugar from the circulation. Thus we see that fundamentally the ingestion of glucose is closely linked up with the action of endogenous insulin which appears to play the principal rôle. The release of the inorganic phosphates from the circulation for the final utilization of carbohydrate, therefore, mainly depends on insulin.

It appears that during active carbohydrate metabolism the secretion of insulin helps, not only in the release of the inorganic phosphate from the blood but also its combination with the sugar to form a compound (hexose-phosphates), in which intermediary stage glucose is finally known to be utilized.

Now, the power of secreting insulin under hyperglycæmic stimulation of the pancreas naturally varies in normal and diabetic subjects. Thus, in healthy normal subjects (under the hyperglycæmic stimulation caused by glucose ingestion), the pancreas is in an efficient condition to secrete the necessary extra amount of insulin and to remove the excess of sugar from the blood stream. In diabetic subjects, however, this power of requisitioning for the necessary extra amount of the hormone after the carbohydrate meal is either deficient or is almost absent depending on the severity of the disease and on the extent of damage to the pancreas. In mild or moderate types of diabetes, though the organ can still produce some hormone, the supply becomes slow and insufficient, thus causing a slower and lesser utilization of sugar and, consequently, slower and lesser demand for the inorganic phosphate of the plasma. In severe cases of diabetes, when the insulin-secreting cells of the pancreas are damaged to such an extent that they can produce very little of the hormone, the shift of the plasma-phosphate becomes almost absent.

If, however, insulin is supplied artificially to these diabetic subjects the inherent deficiency of the hormone is made up temporarily and thus we find a marked change in the behaviour of the shift of the inorganic phosphate in these subjects after insulin injection.

So we see that in mild and moderate cases of diabetes, the decrease in the blood-phosphate value, after the administration of insulin, becomes almost like normal. In severe cases of diabetes, too, there is a sharp initial fall of the phosphate value after insulin injection though the decrease is temporary and lasts for a short period only.

We are, therefore, led to the conclusion that the phosphates play an important part in the intermediary metabolism of carbohydrates and that insulin is a most essential factor in this carbohydrate-phosphate linkage.

SUMMARY.

1. The average inorganic phosphate content of the blood in normal healthy Indian subjects was found to be 3.5 mg. per 100 c.c., the range of variation being 3 mg. to 5 mg. per 100 c.c.
2. Race *per se* does not appear to have much effect on the phosphate content of the blood.
3. The inorganic phosphate content of the blood of diabetic subjects in fasting condition appeared to bear no relationship to the state of the hyperglycæmia.
4. After a glucose feed in a healthy normal person, the blood-phosphate goes on decreasing till the end of one hour and then it begins to rise reaching the initial level by the end of two hours.
5. The effect of glucose feed on the decrease of the phosphate content of the blood in diabetic subjects varies according to the degree of severity of the disease.

their system of blood analysis. The starting point of their system is the preparation of a protein-free blood filtrate suitable for the largest possible number of different determinations. Very small quantities of the filtrate are required for each separate determination. In this system to prepare the protein-free blood filtrate, the blood is first laked with distilled water and the protein then precipitated by means of tungstic acid. It was, however, observed that when the non-protein nitrogenous constituents (urea, uric acid, creatinine, and amino-acids) were estimated separately in this filtrate, the sum of the nitrogen of these constituents was always less than the total non-protein nitrogen determined as such. Thus, the non-protein nitrogen in the whole blood could not be wholly accounted for by the known constituents. Wu's (*loc. cit.*) study on the distribution of the non-protein nitrogenous constituents between corpuscles and plasma threw much light on this discrepancy. Determinations made on the plasma showed that the sum of the nitrogen of the different non-protein nitrogenous constituents agreed fairly closely with the total non-protein nitrogen content, while determinations made on corpuscles showed that the sum of the nitrogen of the different known non-protein nitrogenous constituents was less than the total non-protein content. This discrepancy was similar to the one observed in the case of analysis made on filtrates prepared from the whole blood according to Folin and Wu's technique. It, therefore, became clear that the nitrogen, which could not be accounted for, was contained in the corpuscles.

• Later, Folin (1930*a*) found that it was the laking of blood cells in the preparation of his filtrate which caused the discrepancy noted above. The process of laking disintegrated the red cells and he showed that most of the nitrogen that would not be accounted for in his system of blood analysis came very largely from the disintegrated blood cells. He, therefore, changed his technique of the preparation of the filtrate. To prevent laking of blood cells, he introduced for the dilution of blood a slightly hypertonic solution of sodium sulphate, instead of distilled water. With the new filtrate the total non-protein nitrogen contents of whole blood tallied, within limits of error, with the sum of the nitrogen of the different non-protein nitrogenous constituents.

His new system of blood analysis employing the new method of preparing the blood filtrate and micro methods of analysis is in almost universal use. It was, however, felt worth while to re-examine the question with a view to see if the discrepancy in the analyses on filtrates prepared from laked and unlaked blood in the case of Indian subjects was of the same order as that in the case of American subjects. Blood of 18 normal Indian subjects was examined and the results obtained are given in Tables I, II, and III. Table I gives the figures for the laked filtrates, Table II for the unlaked filtrates made from the same samples of blood, and the average figures are given for comparison in Table III.

It will be noted from the comparative figures in Table III, that as much as 8.30 mg. N per 100 c.c. of blood are left unaccounted for in the laked filtrate results, as compared to only 0.23 mg. N per 100 c.c. of blood in those obtained from unlaked filtrate. These figures conform closely with those obtained by Folin and Svedberg (*loc. cit.*) in the analysis of blood of 19 normal American subjects. In their series, 7.6 mg. of N per 100 c.c. of blood were not accounted for in the case of laked filtrate analysis while only 0.40 mg. N per 100 c.c. was the corresponding figure in the case of analysis of unlaked filtrates.

TABLE I.

Non-protein nitrogenous constituents in the bloods of 18 normal subjects by the laked blood filtrate method.

Number.	Serial number in Table IV.	Diet.	Non-protein nitrogen, mg. per 100 c.c.	Urea nitrogen, mg. per 100 c.c.	Uric acid, mg. per 100 c.c.	Creatinine, mg. per 100 c.c.	Total creatinine, mg. per 100 c.c.	Amino-acid nitrogen, mg. per 100 c.c.
1	3	M	30.00	13.45	3.14	1.26	3.14	7.19
2	8	V	30.00	12.99	2.06	1.43	4.03	7.04
3	23	V	26.43	10.48	3.18	1.34	3.29	6.80
4	28	V	31.91	14.14	2.56	1.25	3.39	6.94
5	29	V	27.27	12.96	2.46	1.63	4.29	6.25
6	48	V	24.69	12.05	2.46	1.36	3.06	6.21
7	62	M	32.79	14.08	3.42	1.20	2.92	7.24
8	63	V	31.58	13.30	2.36	1.43	3.66	7.09
9	67	M	25.32	11.94	3.50	1.13	2.43	6.13
10	78	V	32.61	13.15	2.36	1.41	4.00	7.41
11	79	M	34.88	13.64	2.72	1.31	3.03	8.12
12	80	M	28.57	12.62	3.17	1.34	3.61	6.89
13	81	M	35.71	14.29	3.18	1.36	3.28	7.57
14	94	V	32.79	12.85	3.19	1.47	3.92	7.51
15	107	V	26.91	12.20	3.04	1.42	3.32	6.41
16	108	V	26.09	12.45	2.96	1.23	2.60	6.08
17	115	M	30.91	14.46	2.72	1.29	2.70	6.84
18	116	V	27.40	12.65	3.44	1.61	4.17	6.13
AVERAGE ..			30.38	12.98	2.88	1.36	3.38	6.88

In the diet column, V stands for vegetarian diet and M for mixed diet.

TABLE II.

Non-protein nitrogenous constituents in the bloods of 18 normal subjects by the unlaked blood filtrate method. The same samples of blood were used for these determinations as were used for the determinations referred in Table I.

Number.	Serial number in Table IV.	Diet.	Non-protein nitrogen, mg. per 100 c.c.	Urea nitrogen, mg. per 100 c.c.	Uric acid, mg. per 100 c.c.	Creatinine, mg. per 100 c.c.	Total creatinine, mg. per 100 c.c.	Amino-acid nitrogen, mg. per 100 c.c.
1	3	M	19.02	13.36	2.96	1.42	1.96	3.34
2	8	V	17.55	12.43	2.02	1.22	1.80	2.98
3	23	V	15.17	9.93	3.15	1.10	1.51	3.68
4	28	V	20.56	13.52	2.59	1.14	1.70	4.76
5	29	V	18.63	13.09	2.36	1.16	1.56	3.94
6	48	V	17.13	11.96	2.36	1.03	1.18	3.92
7	62	M	18.93	13.30	3.20	1.04	1.22	3.92
8	63	V	18.46	12.89	2.36	1.16	1.56	3.88
9	67	M	17.20	11.91	2.99	1.01	1.10	3.90
10	78	V	18.44	12.94	2.20	1.12	1.42	3.84
11	79	M	18.45	13.15	2.69	1.14	1.39	3.42
12	80	M	17.73	12.43	3.06	1.16	1.42	3.61
13	81	M	20.45	13.43	3.03	1.07	1.19	4.97
14	94	V	17.81	12.62	3.03	1.14	1.31	3.98
15	107	V	17.08	12.13	2.86	1.15	1.44	3.61
16	108	V	18.47	12.17	2.65	1.05	1.31	4.38
17	115	M	18.94	13.58	2.66	1.14	1.31	4.31
18	116	V	18.20	12.55	3.21	1.14	1.34	4.28
AVERAGE ..			18.23	12.63	2.74	1.13	1.43	3.93

In the diet column, V stands for vegetarian diet and M for mixed diet.

TABLE III.

Giving comparison of average figures of non-protein nitrogenous constituents in the bloods of 18 normal subjects obtained by analysis on laked and unlaked blood filtrates.

Constituent.	MG. N PER 100 C.C.	
	Laked filtrate.	Unlaked filtrate.
Urea nitrogen	12.98	12.63
Uric acid nitrogen	0.96	0.91
Total creatinine nitrogen	1.26	0.53
Amino-acid nitrogen	6.88	3.93
Total N. P. N. by addition	22.08	18.00
Total N. P. N. by estimation	30.38	18.23
Difference	8.30	0.23

The figures in Tables I, II, and III demonstrate once again that Folin's new technique of the unlaked blood filtrate and his improved methods of determination of the non-protein nitrogenous constituents have made it possible to get a correct idea of the non-protein nitrogen distribution in blood. The use of the new technique is imperative, as Folin and Svedberg (*loc. cit.*) say that 'There will be necessarily a sharp break between the hospital records or the past published records of blood analyses based on aqueous extracts from laked whole blood and corresponding records obtained from unlaked blood. But the conclusion seems inescapable that for nearly every kind of metabolism study, it must be obscuring and misleading to include in the analyses of blood as a transportation system an unknown mixture of residues obtained from the destroyed cellular elements which have no direct connection with the circulating waste products or food products'. 'The unlaked blood extracts', they write, 'are just like plasma extracts which . . . are substantially free from unknown nitrogenous materials'.

In each case the blood was drawn from the cubital vein before the subject had his tea or breakfast. Two milligrams of neutral potassium oxalate (Merek's guaranteed reagent) per cubic centimetre of blood were used to prevent coagulation. The blood sample was mixed thoroughly in an Erlenmeyer flask containing the oxalate. The following were determined: Total non-protein nitrogen, urea nitrogen, uric acid, creatinine, total creatinine and amino-acid nitrogen. For these determinations Folin's methods were used (Folin, 1930b; Folin and Svedberg, *loc. cit.*; Folin, 1933; Folin, 1934). All estimations were done in duplicate.

Table IV gives the results obtained by the study of the bloods of 126 healthy Indian men. The averages are given in Table V.

TABLE IV.

Non-protein nitrogenous constituents in the bloods of 126 normal young men.

Number.	Age.	Diet.	Non-protein nitrogen, mg. per 100 c.c.	Urea nitrogen, mg. per 100 c.c.	Uric acid, mg. per 100 c.c.	Creatinine, mg. per 100 c.c.	Total creatinine, mg. per 100 c.c.	Amino-acid nitrogen, mg. per 100 c.c.
1	18	V	17.56	11.80	2.41	1.10	1.36	3.76
2	19	V	18.66	12.50	3.49	1.10	1.22	3.99
3	20	M	19.02	13.36	2.96	1.42	1.96	3.34
4	21	V	15.60	10.06	2.79	1.27	1.51	3.51
5	21	V	18.09	12.50	3.03	1.20	1.67	3.25
6	21	V	20.00	13.72	2.87	1.28	1.69	4.10
7	21	V	17.24	11.79	3.69	1.24	1.50	3.04
8	21	V	17.55	12.43	2.02	1.22	1.80	2.98
9	21	M	17.73	12.24	2.38	1.18	1.47	3.42
10	21	M	16.57	11.31	3.08	1.16	1.39	3.51
11	21	M	19.55	12.82	2.60	1.28	1.44	4.53
12	22	V	18.45	12.75	3.64	1.22	1.70	3.00
13	22	V	19.25	13.30	3.44	1.33	1.77	4.04
14	22	V	18.00	12.08	3.77	1.27	1.49	3.84
15	22	V	16.21	10.91	2.89	1.30	1.49	3.58
16	22	V	15.55	10.91	2.60	1.14	1.27	3.31
17	22	V	20.41	13.88	2.77	1.20	1.41	4.36
18	22	V	19.03	12.74	2.77	1.18	1.38	4.17
19	22	V	19.44	13.88	3.31	1.14	1.63	3.07
20	22	V	19.74	13.29	2.46	1.28	1.48	4.31
21	22	V	16.60	11.41	3.25	1.09	1.29	3.19
22	22	V	16.21	10.69	3.74	1.02	1.35	3.26
23	22	V	15.17	9.93	3.15	1.10	1.51	3.60

In the diet column, V stands for vegetarian diet and M for mixed diet.

TABLE IV—*contd.*

Number.	Age.	Diet.	Non-protein nitrogen, mg. per 100 c.c.	Urea nitrogen, mg. per 100 c.c.	Uric acid, mg. per 100 c.c.	Creatinine, mg. per 100 c.c.	Total creatinine, mg. per 100 c.c.	Amino-acid nitrogen, mg. per 100 c.c.
24	22	M	18.00	11.95	3.74	1.18	1.40	3.75
25	22	M	17.53	12.01	3.74	1.26	1.51	3.82
26	22	M	19.03	12.89	2.69	1.20	1.64	4.14
27	22	M	18.65	12.38	3.40	1.23	1.55	3.82
28	23	V	20.56	13.52	2.59	1.14	1.70	4.76
29	23	V	18.63	13.09	2.36	1.16	1.58	3.94
30	23	V	18.64	12.89	3.72	1.03	1.32	4.08
31	23	V	17.62	11.76	3.12	1.10	1.33	4.08
32	23	V	17.58	11.20	3.20	1.11	1.31	4.03
33	23	V	19.03	12.57	2.52	1.12	1.29	4.54
34	23	V	17.14	11.22	2.93	1.01	1.18	4.31
35	23	V	19.22	13.01	2.60	1.18	1.49	3.92
36	23	V	16.55	11.47	3.23	1.22	1.53	3.66
37	23	V	20.72	13.89	3.45	1.12	1.51	4.61
38	23	V	18.16	12.14	2.65	1.18	1.61	4.26
39	23	V	17.24	12.13	2.72	1.15	1.64	3.44
40	23	V	17.05	11.91	2.96	1.28	1.57	2.87
41	23	M	17.94	11.51	2.84	0.73	0.98	4.72
42	23	M	16.80	10.94	2.98	1.05	1.24	4.03
43	23	M	16.21	10.50	2.90	1.15	1.21	4.05
44	23	M	18.26	12.30	3.68	1.28	1.46	3.92
45	23	M	19.66	13.44	3.42	1.13	1.34	3.39
46	23	M	18.55	12.25	2.89	1.06	1.34	4.28
47	23	M	19.03	13.29	3.10	1.13	1.36	3.73
48	24	V	17.13	11.96	2.36	1.03	1.18	3.92
49	24	V	18.73	12.25	3.14	1.23	1.42	4.30

In the diet column, V stands for vegetarian diet and M for mixed diet.

TABLE IV—*contd.*

Number.	Age.	Diet.	Non-protein nitrogen, mg. per 100 c.c.	Urea nitrogen, mg. per 100 c.c.	Uric acid, mg. per 100 c.c.	Creatinine, mg. per 100 c.c.	Total creatinine, mg. per 100 c.c.	Amino-acid nitrogen, mg. per 100 c.c.
50	24	V	16.59	10.73	3.33	1.26	1.66	4.10
51	24	V	17.63	12.31	2.48	1.23	1.54	3.36
52	24	V	18.10	12.02	2.74	1.68	2.07	3.92
53	24	V	16.22	11.68	3.27	1.04	1.39	3.20
54	24	M	19.20	12.78	2.84	1.01	1.31	4.31
55	24	M	17.93	11.58	3.26	0.99	1.31	4.07
56	24	M	18.84	13.96	3.33	1.11	1.53	3.77
57	24	M	16.54	11.08	3.19	1.08	1.41	3.07
58	24	M	20.73	14.04	3.79	1.04	1.24	4.17
59	24	M	18.96	13.43	3.63	1.48	1.77	4.06
60	24	M	16.53	11.07	2.48	1.06	1.32	3.48
61	24	M	18.22	12.50	2.92	1.21	1.38	3.52
62	24	M	18.93	13.30	3.20	1.04	1.22	3.92
63	25	V	18.46	12.89	2.36	1.16	1.56	3.88
64	25	V	20.23	13.97	2.59	1.11	1.46	4.33
65	25	V	17.07	11.21	2.59	1.26	1.56	3.45
66	25	V	16.06	11.06	3.06	1.23	1.62	3.08
67	25	M	17.20	11.91	2.99	1.01	1.10	3.90
68	25	M	20.09	14.53	3.38	1.09	1.19	4.27
69	25	M	18.27	12.56	2.94	1.16	1.41	4.13
70	25	M	21.92	14.44	2.96	1.11	1.48	4.98
71	25	M	20.38	13.52	3.44	1.13	1.46	4.00
72	25	M	18.74	12.70	3.23	1.23	1.69	3.73
73	25	M	17.13	11.83	3.02	1.23	1.71	3.66
74	25	M	17.22	11.96	3.04	1.00	1.23	3.58
75	25	M	19.82	13.44	3.82	1.05	1.18	4.23

In the diet column, V stands for vegetarian diet and M for mixed diet.

TABLE IV—*contd.*

Number.	Age.	Diet.	Non-protein nitrogen, mg. per 100 c.c.	Urea nitrogen, mg. per 100 c.c.	Uric acid, mg. per 100 c.c.	Creatinine, mg. per 100 c.c.	Total creatinine, mg. per 100 c.c.	Amino-acid nitrogen, mg. per 100 c.c.
76	26	V	21.00	14.35	3.28	1.29	1.56	4.05
77	26	V	17.85	11.95	3.05	1.05	1.25	4.19
78	26	V	18.44	12.94	2.20	1.12	1.42	3.84
79	26	M	18.45	13.15	2.69	1.14	1.39	3.42
80	26	M	17.73	12.43	3.06	1.16	1.42	3.61
81	26	M	20.45	13.43	3.03	1.07	1.19	4.97
82	26	M	17.85	12.63	2.98	1.05	1.36	4.06
83	26	M	19.03	12.56	2.90	1.18	1.50	4.54
84	26	M	18.92	13.36	2.69	1.05	1.38	3.42
85	26	M	18.42	12.50	3.13	1.15	1.26	4.14
86	26	M	17.57	12.08	2.77	1.19	1.52	3.32
87	26	M	20.48	14.13	3.49	1.29	1.68	4.06
88	26	M	18.75	13.80	2.98	1.24	1.63	3.07
89	26	M	17.54	11.80	2.84	1.08	1.48	3.45
90	26	M	19.86	13.58	2.73	1.28	1.67	4.20
91	26	M	18.48	13.88	3.59	1.11	1.44	2.71
92	26	M	18.49	13.44	3.04	1.18	1.44	3.52
93	26	M	19.55	13.96	2.64	1.20	1.36	3.49
94	27	V	17.81	12.62	3.03	1.14	1.31	3.98
95	27	V	17.59	11.96	3.75	1.39	1.68	3.94
96	27	V	16.76	11.52	2.59	1.20	1.38	3.60
97	27	V	19.11	13.16	3.39	1.22	1.46	3.99
98	27	M	20.43	13.93	2.99	1.13	1.36	4.52
99	27	M	21.01	14.45	2.96	1.18	1.39	4.27
100	27	M	18.03	13.02	2.33	1.09	1.34	3.86
101	27	M	21.00	14.20	3.25	1.31	1.61	4.33

In the diet column, V stands for vegetarian diet and M for mixed diet.

TABLE IV—*concl'd.*

Number.	Age.	Diet.	Non-protein nitrogen, mg. per 100 c.c.	Urea nitrogen, mg. per 100 c.c.	Uric acid, mg. per 100 c.c.	Creatinine, mg. per 100 c.c.	Total creatinine, mg. per 100 c.c.	Amino-acid nitrogen, mg. per 100 c.c.
102	27	M	20.43	14.04	3.65	1.38	1.63	4.14
103	27	M	19.80	13.59	3.55	1.09	1.29	4.00
104	27	M	19.80	13.73	2.94	1.27	1.61	4.09
105	27	M	18.55	12.74	2.79	1.14	1.28	3.73
106	28	V	16.56	10.55	2.50	1.12	1.24	4.18
107	28	V	17.08	12.13	2.86	1.15	1.44	3.61
108	28	V	18.47	12.17	2.65	1.05	1.31	4.38
109	28	M	20.54	14.36	3.54	1.17	1.29	4.58
110	28	M	18.00	11.79	3.54	1.21	1.64	3.78
111	28	M	20.08	13.30	2.63	1.18	1.58	4.36
112	28	M	18.53	12.75	3.17	1.25	1.47	3.75
113	28	M	20.10	13.67	3.12	1.24	1.54	4.09
114	28	M	18.18	12.62	2.99	1.20	1.38	3.97
115	28	M	18.94	13.58	2.66	1.14	1.31	4.31
116	29	V	18.20	12.55	3.21	1.14	1.34	4.28
117	29	V	21.54	14.70	3.15	1.41	2.00	4.18
118	29	V	17.53	11.16	3.15	1.30	1.66	4.15
119	29	V	20.09	14.51	2.74	1.27	1.43	4.07
120	29	M	15.18	8.96	3.14	0.91	1.20	4.05
121	29	M	18.94	13.30	2.58	1.16	1.39	3.85
122	30	M	16.06	10.13	3.03	1.15	1.38	4.08
123	30	M	20.53	14.53	2.94	1.03	1.27	4.03
124	32	M	18.91	13.96	3.10	1.05	1.28	3.38
125	32	M	17.01	11.74	2.86	1.07	1.26	3.27
126	32	M	19.70	13.73	3.12	1.21	1.42	3.82
AVERAGE ..			18.43	12.56	3.02	1.17	1.45	3.88

In the diet column, V stands for vegetarian diet and M for mixed diet.

TABLE V.

Averages obtained for the non-protein nitrogenous constituents in the blood by the study of 126 healthy young men.

		MG. PER 100 C.C.	
			In terms of nitrogen.
Non-protein nitrogen	..	18.43	..
Urea nitrogen	..	12.56	12.56
Uric acid	..	3.04	1.01
Creatinine	..	1.17	..
Total creatinine	..	1.45	0.54
Amino-acid nitrogen	..	3.88	3.88

Total N. P. N. by addition	17.99
Total N. P. N. by estimation	18.43

Difference	0.44

NON-PROTEIN NITROGEN.

The average for 126 subjects is 18.43 mg. per 100 c.c. of blood. The frequency distribution of the non-protein nitrogen in the subjects is given in Graph 1. The values range from 15.17 mg. to 21.92 mg. The mean (18.426 ± 0.082) and the median (18.455 ± 0.103) are very close. The standard deviation is 1.369 ± 0.0581 and the co-efficient of variation is 7.428 ± 0.317 . The significant variation is 17.06 to 19.80 covering 66 per cent of the subjects.

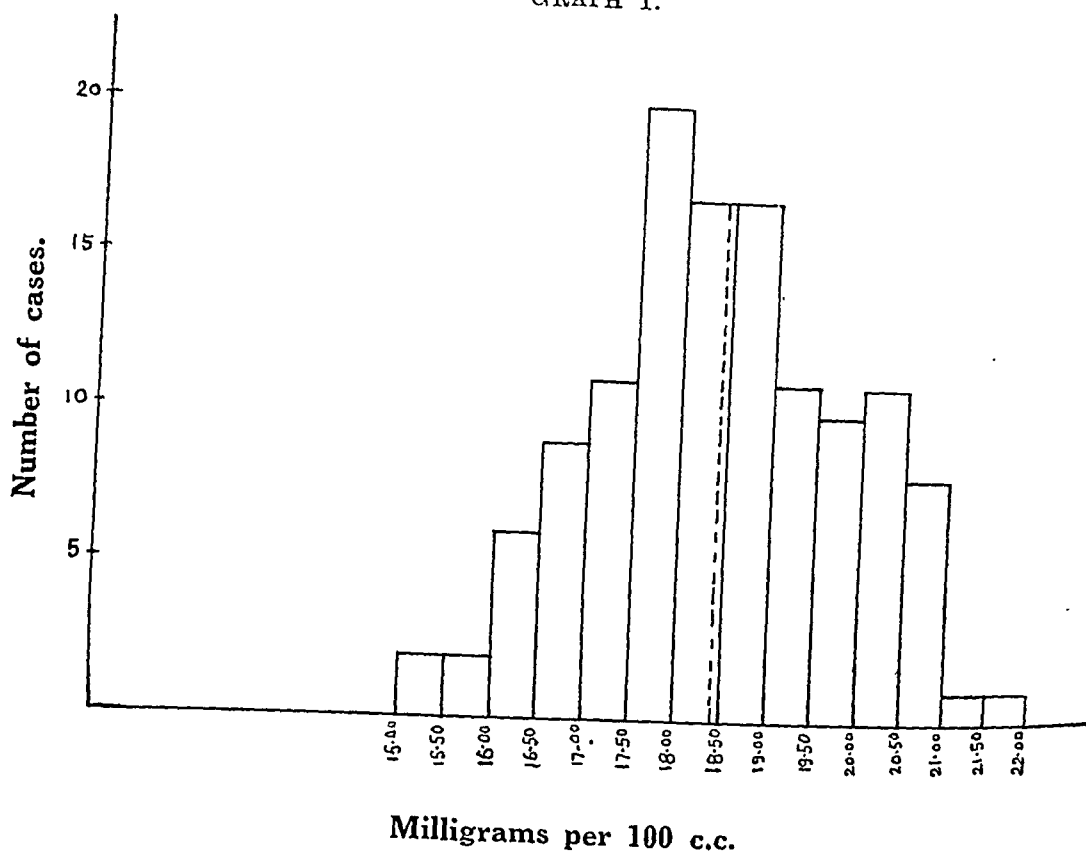
The only data in literature directly comparable to the figures given above are those given by Folin and Svedberg (*loc. cit.*) who using unlaked blood filtrates found an average of 18.5 mg. per 100 c.c. with a range of 13.8 mg. to 20.8 mg. for 19 normal Americans. These workers do not give the age range of their subjects but merely say they were students. They at the same time had also made determination of non-protein nitrogen on the same samples using laked blood filtrates. In this case their average for the 19 subjects was 30.7 mg. (range 25.6 mg. to 42.0 mg.) per 100 c.c.

The rest of the data for total non-protein nitrogen content of blood available in literature is that obtained from analysis of laked blood filtrates, e.g., Hawk and Bergeim (1938); Berglund (1922). The values generally range from 25 mg. to 35 mg. per 100 c.c. with an average of about 30 mg. These figures compare with my average of 30.38 mg. (range 24.69 mg. to 35.71 mg.) per 100 c.c., obtained by

the analysis of blood of 18 normal Indians by the use of laked blood filtrates (Table I). Unlaked blood filtrates from the same samples of blood of these 18 subjects gave for the non-protein nitrogen content an average of 18.23 mg. (range 15.17 mg. to 20.56 mg.) per 100 c.c.

It will be thus seen that the values for the non-protein nitrogen content in unlaked blood filtrates (18.5 mg. and 18.23 mg.) are lower than those obtained in laked blood filtrates (30.7 mg. and 30.38 mg.). They are lower by about 12 mg. per 100 c.c. This large difference in the two figures for the non-protein nitrogen

GRAPH 1.



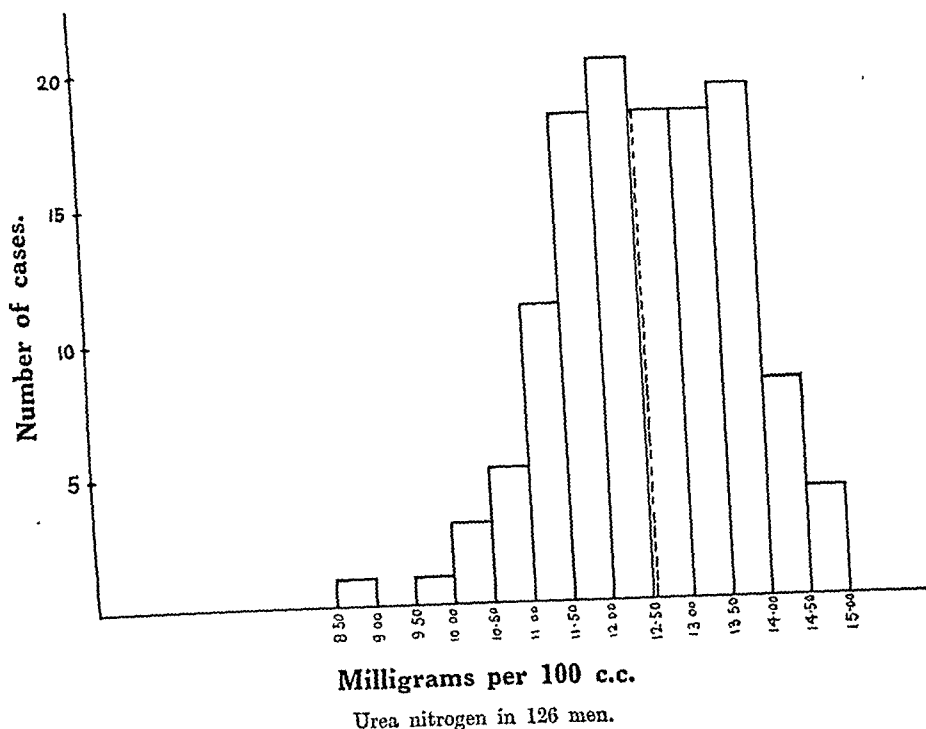
Non-protein nitrogen in 126 men.

content, termed as undetermined or rest nitrogen, results from the destruction of the cells in the process of laking and has nothing to do with the process of metabolism. When laking is prevented, this undetermined or rest nitrogen disappears as is shown in Tables I, II, and III. It is evident, therefore, that the use of the unlaked blood filtrate is imperative if any study of the non-protein nitrogen metabolism in blood is to be made. Inclusion of material from the disintegration of the cells in the blood filtrate, as happens in the laked blood filtrate method, will not give a correct idea of the non-protein nitrogen metabolism in blood.

UREA NITROGEN.

The average urea nitrogen content for the 126 subjects studied is 12.56 mg. per 100 c.c. of blood. The values range between 8.96 mg. and 14.70 mg. per 100 c.c. The frequency distribution is given in Graph 2. The mean and the median are 12.558 ± 0.0681 and 12.569 ± 0.0852 respectively. The standard deviation is 1.137 ± 0.0483 and the co-efficient of variation is 9.053 ± 0.388 . The significant variation is 11.42 to 13.70 and covers 66 per cent of the subjects.

GRAPH 2.



Folin and Svedberg (*loc. cit.*) using unlaked filtrate found an average of 12.6 mg. per 100 c.c. with a range of 9.4 mg. to 14.2 mg. for their series of normal subjects.

A large number of other data are available in literature but these were obtained by the use of laked blood filtrates. Folin and Svedberg (*loc. cit.*) themselves determined on their normal subjects urea nitrogen in laked blood filtrates and obtained an average of 13.0 mg. per 100 c.c. with a range of 9.8 mg. to 14.5 mg. Berglund (*loc. cit.*) gives an average of 12.0 mg. per 100 c.c. (range 9 mg. to 15 mg.), while Hawk and Bergeim (*loc. cit.*) give a range of 10 mg. to 15 mg. per 100 c.c. MacKay and MacKay (1927), summarizing available data in literature together with data of their own, give values ranging from 5 mg. to 23 mg. of urea nitrogen with an

average of 15.4 mg. per 100 c.c., but the great majority of their values fall between 8 mg. and 18 mg. per 100 c.c. Priestley and Hindmarsh (1924, quoted by Peters and van Slyke, 1931) found an average blood urea nitrogen content of 11.3 mg. per 100 c.c. of blood. My analysis of 18 bloods of normal Indians done on laked filtrate (Table I) gives an average of 12.98 mg. (range 10.48 mg. to 14.46 mg.) per 100 c.c. Unlaked blood filtrates from the same samples of blood of these 18 subjects gave an average of 12.63 mg. (range 9.93 mg. to 13.38 mg.) per 100 c.c.

It will be observed that the urea nitrogen content when estimated in unlaked filtrate does not materially differ from that obtained in laked blood filtrate (compare 12.63 mg. with 12.98 mg.), but the total non-protein nitrogen content of laked filtrates is higher by 12 mg. per 100 c.c. Therefore, for unlaked blood, the urea nitrogen represents about 68 to 69 per cent of the total non-protein nitrogen, whereas for laked blood it represents only about 42 per cent.

RATIO BETWEEN TOTAL URINARY NITROGEN AND BLOOD UREA NITROGEN.

Ever since the methods for the determination of urea nitrogen in blood and urine came into vogue efforts have been made to establish a relation between concentration of nitrogenous products in urine and in blood. Earlier workers, e.g., McLean and Selling (1914), tried to establish a relation between concentration of urea in blood and in urine on the lines of Ambard's co-efficient. They have given data for 10 normal individuals. Their results give an average total urinary nitrogen in 24 hours of 10.02 g. and an average blood urea nitrogen content of 14.95 mg. per 100 c.c. From these figures a ratio between total urinary nitrogen in grammes and blood urea nitrogen in mg. per 100 c.c. will be $\frac{10.02}{14.95} = \frac{1}{1.5}$ approximately

Later, Peters and van Slyke (*loc. cit.*) worked out a relation between total urinary nitrogen and blood urea nitrogen, basing their observation on the results obtained by Priestley and Hindmarsh (*loc. cit.*) and MacKay and MacKay (*loc. cit.*). They wrote 'From the data of MacKay and MacKay (1927) and of Priestley and Hindmarsh (1924) one may conclude that in normal subjects the ratio $\frac{\text{abolized per day}}{\text{per 100 c.c. of blood}}$ averages approximately 1:1'. The data of MacKay and MacKay referred to by Peters and van Slyke show that in men fed 1.1 grammes of protein per kilo per day, the range of blood urea contents was found to be from 9 mg. to 17 mg. of urea nitrogen. MacKay and MacKay have not given actual figures of their results, but from their graphs depicting their results, it seems that an average blood urea nitrogen content of about 12 mg. per 100 c.c. and average total urinary nitrogen content of about 11 grammes in 24 hours have been obtained by them for their six normal men fed on 1.1 g. of protein per kilo per day.

While a ratio of 1 to 1.5 was obtained from the results of determinations by McLean and Selling as quoted above, a ratio of 1:1 was arrived at by Peters and van Slyke on the basis of results of Priestley and Hindmarsh and MacKay and MacKay. Results obtained here on normal Indian subjects (Table VI) do not confirm either of the above two ratios. The average total urinary nitrogen in 24 hours for the normal Indian subjects studied is 5.59 and the average blood urea nitrogen is 11.94 mg. per 100 c.c. Thus,

the ratio between the total urinary nitrogen in grammes and the blood urea nitrogen in 100 c.c. will in the case become $\frac{5.59}{11.94} = 1:2$ approximately. The diet of the Indians differs from that of the American or Australian subjects. The total urinary nitrogen excretion in 24 hours of Indian subjects is generally below 7 g., while that of the American subjects is generally over 12 g. The urea nitrogen contents of blood, however, do not so differ in the two cases, being about 12 mg. per 100 c.c.

It appears that a definite ratio does not exist between the total urinary nitrogen and the blood urea nitrogen as the urinary nitrogen concentration depends on the nitrogen content of diet. A diet, less rich in meat proteins, will give a lower urinary nitrogen excretion: but it will not lower the non-protein or urea nitrogen content in blood. Folin (1917), while discussing the blood non-protein nitrogen and urea nitrogen levels, remarked 'Nor are these levels materially affected by reasonable variations in the nitrogen content of the food. . . . One interesting aspect of the non-protein nitrogen and urea problem is the question as to the effect of the food protein on the level maintained. In the case of strictly normal persons it makes practically no difference whether the diet is rich or poor in nitrogen'. It is only extremely low protein intake, as found by Smith (1926), that will cause a lowering of blood non-protein nitrogen. In normal persons consuming adequate protein, non-protein nitrogen or urea nitrogen content will remain materially unchanged in blood whether the diet is a high protein or a low protein one. Such a difference in the diet will only cause a difference in the total urinary nitrogen.

TABLE VI.

Total nitrogen excretion in 24 hours' collections of urine and urea nitrogen content in blood in 6 normal subjects.

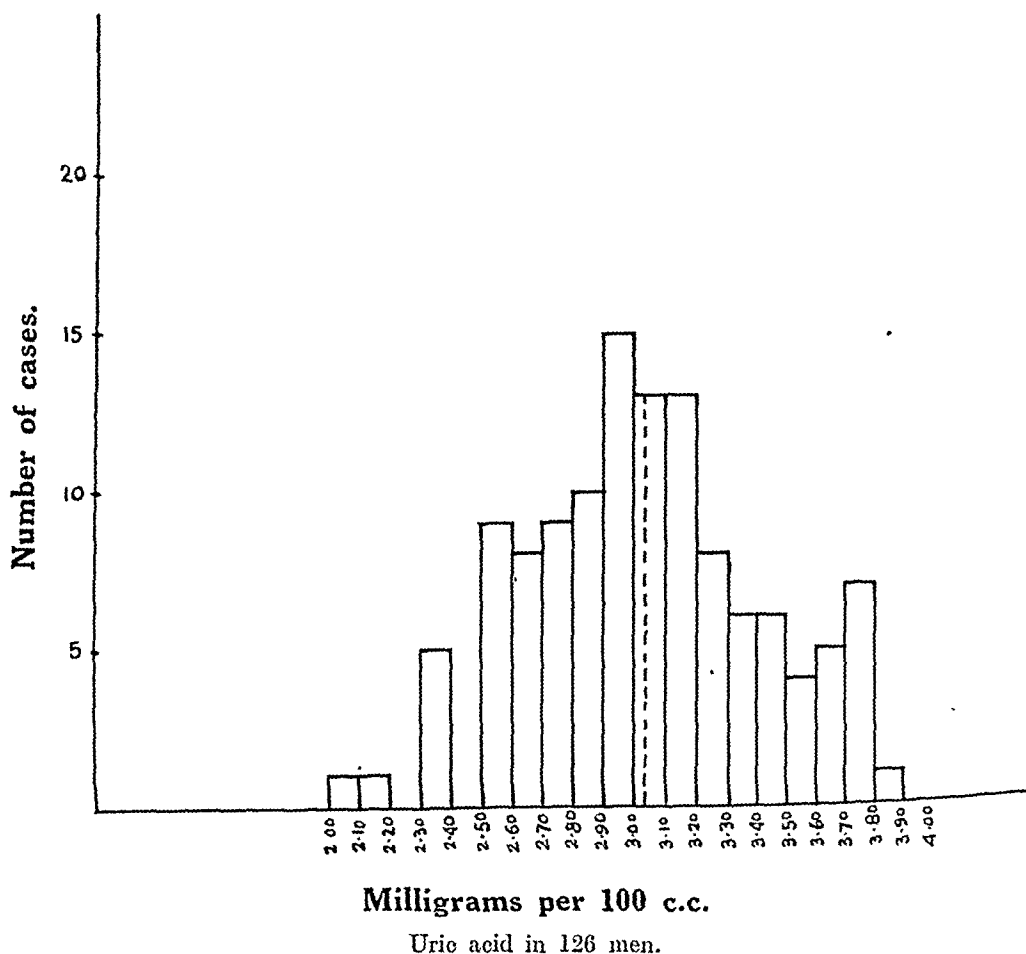
Number.	Total nitrogen in 24 hours' urine, grammes.	Blood urea nitrogen, mg. per 100 c.c.
1	5.01	12.14
2	5.84	13.50
3	6.14	9.80
4	3.62	12.87
5	6.66	13.04
6	6.26	10.31
AVERAGE ..	5.59	11.94

URIC ACID.

The average obtained for the 126 normal subjects examined is 3.02 mg. per 100 c.c. of blood. The frequency distribution of the values obtained is shown in Graph 3. The values range between 2.02 mg. and 3.82 mg. per 100 c.c. of blood. The mean 3.023 ± 0.0236 and the median 3.033 ± 0.0301 are quite

close. The standard deviation is 0.395 ± 0.0167 and the co-efficient of variation is 13.21 ± 0.571 . The significant variation is 2.63 to 3.42 and covers 67 per cent of the subjects examined. The 3.02 mg. of uric acid correspond to 1.007 mg. of uric acid nitrogen when expressed in terms of nitrogen.

GRAPH 3.



Folin and Svedberg (*loc. cit.*) working with unlaked filtrate obtained an average of 2.6 mg. per 100 c.c. with a range of 2.2 mg. to 3.2 mg. for their normal bloods.

One finds in literature figures for laked blood filtrates varying from 2.5 mg. to 5.0 mg. per 100 c.c. for uric acid content of normal human blood (Peters and van Slyke, *loc. cit.*). These figures were based on the use of the old methods of Folin and Wu (*loc. cit.*) and Benedict (1922). Later modifications of these earlier methods and improved uric acid reagent gave a range of 2 mg. to 3.5 mg. per 100 c.c. (Hawk and Bergeim, *loc. cit.*). Folin and Svedberg (*loc. cit.*) again improved the method and introduced a specially sensitive uric acid reagent. Using this improved method they have determined uric acid in the laked blood

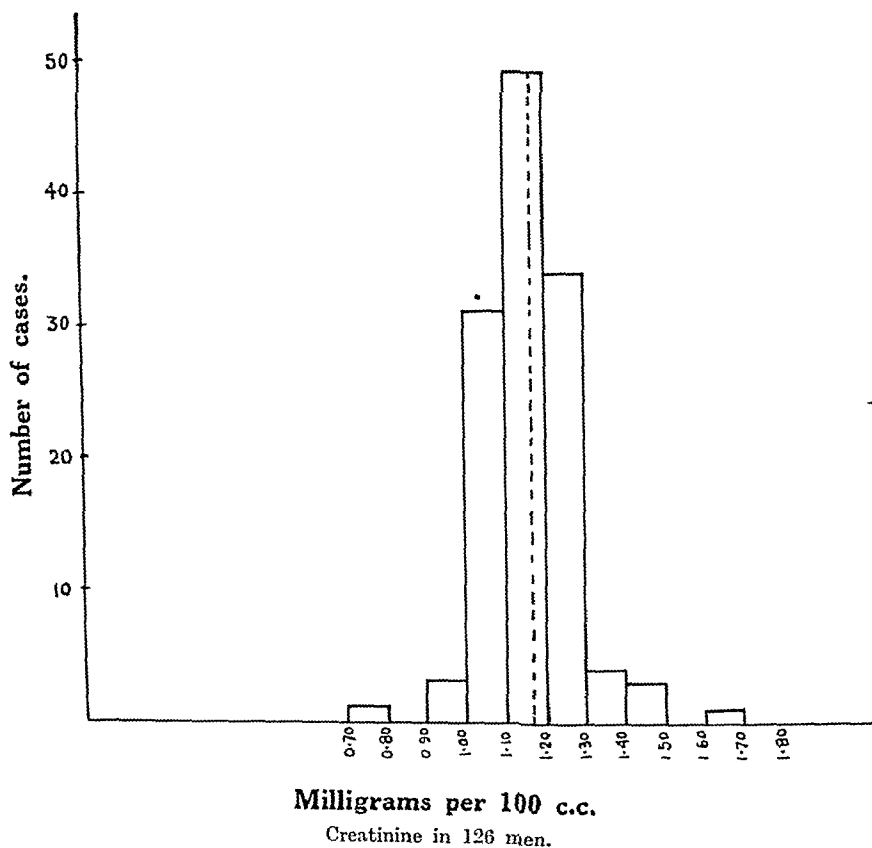
filtrates of their normal subjects and obtained an average of 2.5 mg. with a range of 2.2 mg. to 3.3 mg. per 100 c.c.

The analysis of the laked filtrates of the bloods of my series of the 18 normal Indians gave an average of 2.88 mg. to 3.50 mg. (Table I). Unlaked blood filtrates from the same samples of blood of these 18 subjects gave an average uric acid content of 2.74 mg. per 100 c.c. with a range of 2.02 mg. to 3.21 mg. (Table II). These results show that the uric acid content in unlaked blood filtrate does not appreciably differ from that in laked blood filtrate.

CREATININE.

The average creatinine content in the 126 normal subjects studied is 1.17 mg. per 100 c.c. of blood. The values ranged between 0.73 mg. and 1.68 mg. The

GRAPH 4.



frequency distribution of the values obtained is shown in Graph 4. The mean 1.166 ± 0.0065 and the median 1.172 ± 0.0081 are quite close. The standard

deviation is 0.109 ± 0.0046 and the co-efficient of variation is 9.348 ± 0.401 . The significant variation is 1.06 to 1.28. Seventy-five per cent of the subjects studied fall within the range of significant variation. Ninety per cent of the values are between 1.01 and 1.30, thus clustering near the average of 1.17 mg.

Folin and Svedberg (*loc. cit.*) using unlaked filtrates obtained an average of 1.09 mg. per 100 c.c. with a range of 1.03 mg. to 1.20 mg. for their normal bloods.

Peters and van Slyke (*loc. cit.*) and Hawk and Bergeim (*loc. cit.*) give for laked blood filtrates a range of 1 mg. to 2 mg. per 100 c.c. for creatinine content in normal human blood. Folin and Svedberg (*loc. cit.*) obtained by analysing laked filtrates of the bloods of their normal subjects an average of 1.38 mg. (range 1.20 mg. to 1.45 mg.) per 100 c.c. My series of 18 subjects gave for laked blood filtrates an average creatinine content of 1.36 mg. (range 1.20 mg. to 1.63 mg.) per 100 c.c. (Table I). Unlaked blood filtrates from the samples of blood of these subjects gave an average of 1.13 mg. (range 1.01 mg. to 1.42 mg.) per 100 c.c. (Table II).

It will be observed that the values for creatinine content obtained for unlaked blood filtrates are lower than those obtained for laked blood filtrates. This is because the laked blood filtrate as a result of the disintegration of the corpuscles contains substances which react with the alkaline picrate reagent.

Danielson (1936) and Miller and Dubos (1937) have shown that the erythrocytes contain large amounts of the non-creatinine material and that the non-creatinine material constitutes only a small fraction of the total chromogenic material in the normal plasma. Miller and Dubos (*loc. cit.*) remark ' . . . it seems desirable to discontinue the analysis of whole blood filtrates for "apparent creatinine" and to restrict such analyses entirely to plasma or serum. The results would then be a fairly good approximation of the true values in normal individuals. If whole blood must be employed, then it would seem preferable to prepare the filtrate by a technique (Folin, 1930b) which obviates the laking of the erythrocytes. Creatinine values obtained by this technique agree reasonably well with those found by us for true creatinine in normal plasma '.

TOTAL CREATININE.

Total creatinine is the preformed creatinine plus the creatinine corresponding to creatine. The creatine is transformed to creatinine by the action of dilute hydrochloric acid in an autoclave and then the total creatinine is determined. Subtracting the amount of the preformed creatinine gives the amount of creatinine corresponding to the creatine.

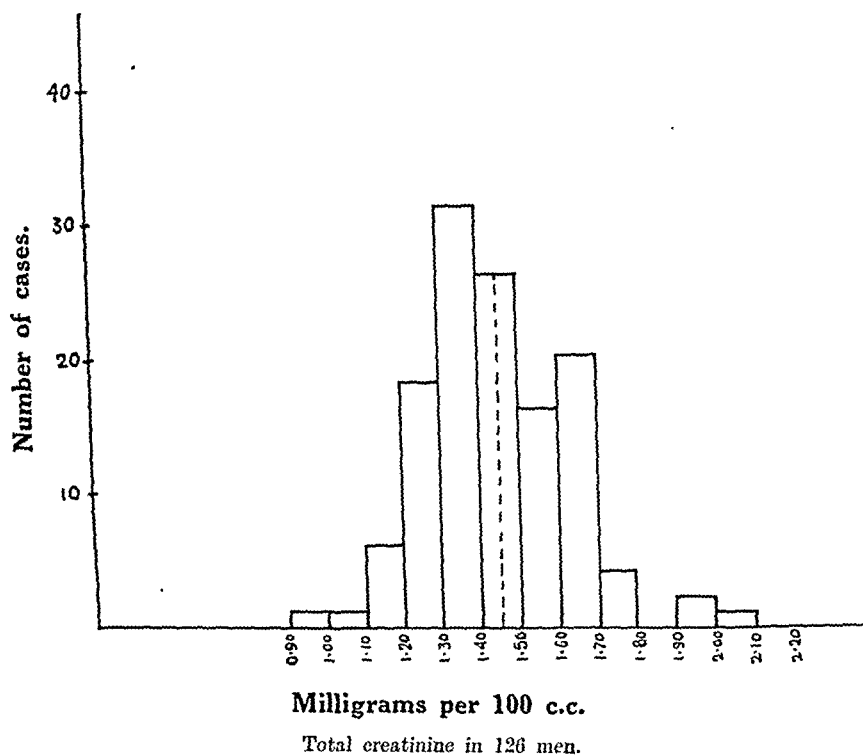
The average obtained for the total creatinine content for the 126 subjects studied is 1.45 mg. per 100 c.c. of blood. The values range between 0.98 and 2.07 mg. Graph 5 gives the frequency distribution of the values obtained for the subjects studied. The mean is 1.448 ± 0.0107 and the median is 1.464 ± 0.0134 . The standard deviation is 0.179 ± 0.0076 and the co-efficient of variation is 12.36 ± 0.533 . The significant variation is 1.27 to 1.63 and covers 70 per cent of the subjects.

The average content of preformed creatinine for the 126 subjects studied was 1.17 mg. per 100 c.c. Subtracting 1.17 mg., the value of the preformed creatinine, from 1.45 mg., the average value obtained for total creatinine leaves

0.28 (1.45 mg. — 1.17 mg.) per 100 c.c. for the creatine content. But this value is in terms of creatinine. Expressed as creatine, this 0.28 mg. will become 0.28×131 (mol. weight of creatine) \div 113 (mol. weight of creatinine) = 0.32 mg.

Figures given in literature for creatine content in blood are those obtained from laked blood filtrates. Hammett (1920) gives an average creatine content of 3.59 mg. (1.30 mg. creatine nitrogen = 3.59 mg. creatine) per 100 c.c. Peters and van Slyke (*loc. cit.*) have quoted values varying from 1.5 mg. to 7 mg. per 100 c.c. for creatine concentration in normal human blood. They remark 'the inaccuracy and lack of specificity of analytical procedures may in part explain this great variability'.

GRAPH 5.



Hawk and Bergeim (*loc. cit.*) give a range of 4 mg. to 5 mg. per 100 c.c. The analysis of laked filtrates of the bloods of my series of 18 normal Indian subjects (Table I) gave an average creatine content of 2.34 mg.* per 100 c.c. Unlaked filtrates

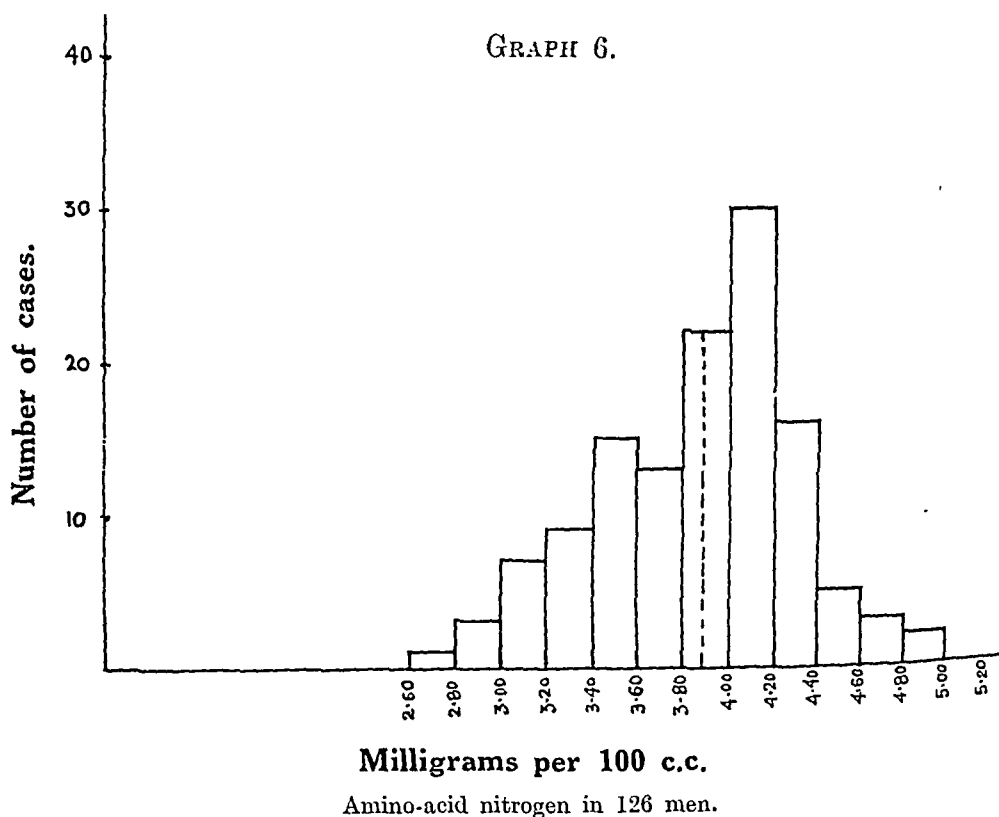
* Subtracting the creatine average of 1.36 mg. from the total creatinine average of 3.38 mg. gives 2.02 mg. as the value for creatine in terms of creatinine. 2.02 mg. become 2.34 mg. when expressed in terms of creatine: $\frac{2.02 \times 131}{113}$

from the same samples of blood of these 18 subjects (Table II) gave an average of 0.35 mg.* per 100 c.c.

These figures show that the laked blood filtrates contain much more creatine than the unlaked blood filtrates. This is due to the disintegration of the corpuscles. The unlaked blood filtrate contains a very small portion of the creatine content of the whole blood, because creatine in the blood is mainly contained in corpuscles as has been shown by various workers, e.g., Hunter and Campbell (1917, 1918), Wilson and Plass (1917) and Wu (*loc. cit.*). Wu by direct analysis of the corpuscles and plasma obtained a creatine value of 6.77 mg. per 100 c.c. for corpuscles and 0.27 mg. per 100 c.c. for plasma, respectively. The low values for the creatine content in the unlaked blood filtrate also confirm these observations.

AMINO-ACID NITROGEN.

The average amino-acid nitrogen content in blood for the 126 normal subjects examined is 3.88 mg. per 100 c.c. of blood. The values obtained



range between 2.71 mg. and 4.98 mg. Graph 6 gives the frequency distribution of the values obtained. The mean 3.877 ± 0.0261 and the median

* Subtracting the creatine average of 1.13 mg. from the total creatinine average of 1.43 mg. gives 0.30 mg. as the creatine value in terms of creatinine. Expressed as creatine, it becomes 0.35 mg. (0.30×1.31).

3.941 ± 0.0327 are close. The standard deviation is 0.437 ± 0.0186 and the co-efficient of variation is 11.27 ± 0.485 . The significant variation is 3.44 and covers 70 per cent of the subjects.

For unlaked blood filtrates Folin and Svedberg (*loc. cit.*) found for their 19 normal subjects an average amino-acid nitrogen content of 5.0 mg. (range 4.2 mg. to 5.6 mg.) per 100 c.c. Danielson (1933) gives an average of 3.0 mg. per 100 c.c. with a range of 2.3 mg. to 3.73 mg. for unlaked blood filtrates for his series of 29 young men.

Other data given in literature are based on analysis of laked blood filtrates. Peters and van Slyke (*loc. cit.*) and Hawk and Bergeim (*loc. cit.*) quote a range of 5 mg. to 8 mg. per 100 c.c. for the amino-acid nitrogen content in normal human blood. Folin and Svedberg (*loc. cit.*) found by analysis of laked blood filtrates of their normal subjects an average of 8.7 mg. (range 7.8 mg. to 10.2 mg.) per 100 c.c. Analysis of laked filtrates of the blood of 18 of my series of normal subjects (Table I) gave an average of 6.88 mg. (range 6.08 mg. to 8.12 mg.) per 100 c.c. Unlaked blood filtrates from the same samples of blood of these subjects (Table II) gave an average of 3.93 mg. per 100 c.c. with a range of 2.98 mg. to 4.97 mg. Thus, laked blood filtrates give for amino-acid nitrogen content values higher than the unlaked filtrates do. In Danielson's (1933) opinion these higher values in laked blood filtrates are due to the presence in such filtrates of substances contained in red blood cells 'having one or more free amino groups capable of reacting with our reagents and are not capable of diffusing out of the red blood cell'. The unlaked blood filtrate allows expulsion of the interfering substances.

SUMMARY.

1. Standards for the normal averages for the non-protein nitrogen constituents in blood have been worked out by the study of the bloods of 126 healthy young men from the Bombay Presidency between the ages of 18 and 32 years. The findings are tabulated as follows:—

Averages and range of variation in normal findings of 126 healthy young men.

		Mean mg. per 100 c.c.	Minimum mg. per 100 c.c.	Maximum mg. per 100 c.c.	Standard deviation.	Percentage of subjects within significant variation.
Non-protein nitrogen	..	18.43	15.17	21.92	± 1.37	66
Urea nitrogen	..	12.56	8.96	14.70	± 1.14	66
Uric acid	..	3.02	2.02	3.82	± 0.395	67
Creatinine	..	1.17	0.73	1.68	± 0.109	75
Total creatinine	..	1.45	0.98	2.07	± 0.179	70
Amino-acid nitrogen	..	3.88	2.71	4.98	± 0.437	70

These averages are obtained by the analysis of unlaked blood filtrates according to the improved technique of Folin. In the new technique a slightly hypertonic solution of anhydrous sodium sulphate is used for dilution of the blood instead of distilled water, to prevent the laking of the red corpuscles. The filtrate thus obtained is free from material set free by the disintegration of the red cells. The total non-protein nitrogen is completely accounted for by the sum of the amounts of the different non-protein nitrogenous constituents. This was not the case when laked blood filtrates were used for analysis.

2. Samples of bloods were analysed by using both the laked and unlaked filtrates and the results compared. The total non-protein nitrogen, creatinine, total creatinine, and amino-acid nitrogen content values in unlaked filtrates are markedly lower than those obtained in laked filtrates. The uric acid and urea nitrogen contents are almost at the same level, slightly lower values being obtained in unlaked filtrates.

The urea nitrogen content in unlaked blood filtrate analysis is about 68 per cent of the total non-protein nitrogen content, while in the laked filtrates urea nitrogen works up to about 42 per cent of the total non-protein nitrogen.

3. A ratio of 1:2 between total urinary nitrogen excretion in 24 hours in grammes and the blood urea nitrogen in mg. per 100 c.c. is obtained. This differs from the ratio of 1:1 obtained by other workers for European and American subjects. The reason seems to be that, while the blood urea nitrogen obtained from Indian subjects tallies with that for European and American subjects, the total urinary nitrogen excretion is only about half.

ACKNOWLEDGMENTS.

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ABSTINENCE SYMPTOMS IN OPIUM ADDICTION AND THE ROLE OF GLUCOSE IN THEIR TREATMENT.

BY ~

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IN a previous paper Chopra, Mukherjee and Chopra (1935) observed that the percentage of total proteins in the blood sera of opium addicts stands at the lower limit of the range for normal subjects. This they attributed to the hydræmic condition of the blood as a result of the opium habit. In course of their studies on experimental addiction to morphine in dogs, Barbour, Hunter and Richey (1929) also observed a slightly low specific gravity in both blood and serum and also a depression of the water exchanges with the environment. These observations, however, clearly point to an increase in the fluid content of the blood.

On withdrawal of the drug habit it has been observed by Chopra and Chopra (1937) in more than 120 cases of human addicts of long standing that a general dehydration of the body takes place in the form of excessive perspiration, abnormal salivation, and in most of the cases an obstinate diarrhœa. Such observations have also been made by workers like Sollier (1910), Maguin (1909), Pierce and Plant (1928), and others. Some of them have observed an actual polyuria in many addicts on withdrawal. These facts, however, strongly suggest that such a dehydration process may have an ultimate effect on the hydræmic condition of the blood. From our previous observation that a diminished excretion of water during addiction in most of the cases was invariably accompanied by an increase in the fluid content of the blood, we were inclined to believe that an excessive secretion on withdrawal will have a reverse effect leading to an increase in the percentage of serum proteins. In the literature, however, we come across several workers who have observed an increased hydræmic state of the blood on withdrawal leading to a further dilution as compared to pre-withdrawal period. Thus, Barbour, Hunter and Richey (*loc. cit.*) are of opinion that a 'hypersecretion of

morphine withdrawal rather than causing a hypothetical dehydration or "detoxification" may be looked upon as a natural accompaniment of the hydration of blood and probably of the tissues in general'. They, however, explain their conclusions on the hypothesis that 'regardless of diet, addiction diminishes the water intake as well as the water "deficit"'. Withdrawal evokes a decided overcompensation in both the above factors'. Pierce and Plant (*loc. cit.*) also subscribe to a similar opinion.

The recent work of Chopra and Roy (1937) on the lipoids and cholesterol content in the blood sera of opium addicts before, during, and after withdrawal, on the contrary, points to the probability of a diminution in the water content of the blood on withdrawal as we previously supposed. These conflicting views led us to undertake the present work and to actually determine the effect of withdrawal on the blood fluid and also to explain, if possible, the rationale of the treatment of opium addiction by the help of lecithin and glucose that have proved successful in a large majority of cases observed by us. For this reason confirmed opium addicts of long standing were admitted into the Carmichael Hospital for Tropical Diseases, where they were kept under strict supervision. Every precaution was taken to stop the supply of the drug to the subjects by smuggling. The urine of the addicts under treatment were regularly examined for morphine to be sure that the subject was not surreptitiously taking any of the drug. Blood sera from these addicts were examined for total proteins before, during, and after the withdrawal of the drug.

Experimental procedure.—Five c.c. of venous blood were taken and the serum separated from it. The total proteins of these sera were estimated by the micro-refractometric method of Robertson as previously described (Chopra, Mukherjee and Rao, 1934). The serum was examined soon after the admission of the patient, then again three or four days after admission during which period the drug was withheld and withdrawal symptoms were acute. Lastly, they were again examined after the withdrawal symptoms have been relieved by treatment with lecithin. The data obtained are presented in the Table:—

TABLE.

Case number.	PERCENTAGE OF TOTAL PROTEINS.			Case number.	PERCENTAGE OF TOTAL PROTEINS.		
	Before treatment.	Withdrawal period.	After treatment.		Before treatment.	Withdrawal period.	After treatment.
1	7.1	..	6.63	7	7.44	8.01	7.28
2	7.85	8.93	6.69; 7.25	8	7.22	7.89	7.36
3	8.09	7.6	7.39	9	6.93	7.31	7.25
4	9.78	8.87	9.12	10	8.66	9.07	8.82
5	7.75	7.61	..	11	8.37	9.04	8.74; 7.46
6	8.0	7.34	7.53	12	8.97	9.45	8.18; 7.82

TABLE—concl'd.

Case number.	PERCENTAGE OF TOTAL PROTEINS.			Case number.	PERCENTAGE OF TOTAL PROTEINS.		
	Before treatment.	Withdrawal period.	After treatment.		Before treatment.	Withdrawal period.	After treatment.
13	8.47	8.66	8.33 ; 8.10	22	6.84	7.03	..
14	8.20	8.30	8.10	23	7.35	6.73	7.53
15	6.78	7.35	6.97	24	6.82	7.44	7.15
16	7.72	8.29	8.03	25	8.59	8.89	8.74
17	7.52	7.9	7.32	26	8.33	9.00	8.49
18	5.82	6.67	..	27	8.92	9.38	9.2 ; 8.72
19	7.28	6.82	..	28	8.38	8.60	8.24
20	6.96	7.4	6.16	29	8.08	8.28	7.98
21	7.68	8.14	7.45	30	6.52	7.13	6.72

DISCUSSION.

In most of the cases it is found that the concentration of proteins present in serum before treatment undergoes a definite increase during the withdrawal period and returns gradually to almost the pre-withdrawal value after the addict has been thoroughly treated. In a very few cases the percentage of protein during the withdrawal period instead of increasing has suffered a diminution. In such cases, however, the values have not changed very much after treatment. This strongly suggests the presence of certain other factors controlling the total protein content of the blood.

The increase in the total protein content during the period of withdrawal as a general rule runs parallel to the appearance of withdrawal symptoms. Excessive outflow of water from the body is one of the marked withdrawal symptoms that has been observed in almost all the cases studied by us. This apparently points to a disturbance in the fluid equilibrium in general in the body. The drainage of fluid is likely to affect the blood in the long run and the loss of water that the blood has to suffer due to an excessive drainage may increase the percentage of serum proteins. The concentration of other blood constituents may also be affected in a similar way. Barbour, Hunter and Richey (*loc. cit.*) under similar conditions have, on the other hand, observed a general diminution of specific gravity of both blood and serum on withdrawal than during addiction and certain other changes that point to an increase in the blood fluids. In only 4 out of 30 cases we have been able to observe any indication of an increased blood hydration. It will not be out of place to mention here that all our subjects were given to opium addiction for prolonged periods, while Barbour and

co-workers worked with dogs whose period of addiction may be regarded as comparatively short. It is quite likely that this conflicting observation may be the result of a fundamental difference in the period of addiction. This remark of ours may be justified because these workers could not observe any change in specific gravity on longer addiction as compared to normal dog's blood sera. It is quite possible that they would have observed an actual increase of specific gravity on still longer addiction. Absence of any marked withdrawal symptoms in dogs of long addiction as observed by Barbour and co-workers could not, however, be corroborated by us in our human subjects. On the contrary, we observed that in general the longer the period of addiction the more acute the withdrawal symptoms.

From our data taken after treatment it is evident that the proteins have returned almost to their original values. The effect of treatment may, therefore, be taken to have restored the fluid equilibrium of the system to its previous level.

In a previous paper by Chopra, Mukherjee and Chopra (*loc. cit.*) it was indicated that the observed increase of euglobulin probably meant an ultimate drainage of phosphate from the nerve cells. Lecithin treatment was, therefore, suggested on that basis. In the majority of cases lecithin decreased the intensity of withdrawal symptoms and shortened their duration (Chopra and Chopra, *loc. cit.*). But in spite of its administration the abstinence symptoms were very severe in some of the subjects and in these cases intravenous injections of 25 c.c. of 25 per cent glucose helped to ameliorate the condition. Although lecithin was unable to cope with the severity of the withdrawal symptoms it doubtless removed the craving for the drug in the majority of cases (Chopra and Chopra, *loc. cit.*).

However the rôle of glucose in such cases can be understood from our present observations. By treatment the ultimate effect seems to be the restoration of the water balance. Therefore, any drug that confers a fluid retaining power to the blood is expected to have good effect. Carbohydrates in general and glucose in particular are known to have this water retention capacity. Glucose, therefore, in addition to stacking the liver with glycogen to enable it to cope with the unusual strain on this organ during the process of elimination of morphine, helps retention of water in the blood and keeps up the blood hydration level to its normal value. From the above we may, therefore, conclude that lecithin tones up the nerves of the addicts and glucose helps to restore the disturbed water balance, and so it is not difficult to see how these two together produce the desired effect in removing the drug habit and alleviating abstinence symptoms from an opium addict.

SUMMARY AND CONCLUSION.

1. Withdrawal of opium causes an increase in the total proteins in the blood sera, thereby indicating a loss of fluid from the blood.
2. Treatment of opium addiction by means of lecithin and glucose brings the total proteins to their pre-withdrawal level.
3. A rationale of the action of glucose has been put forward.

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TRUE ACHLORHYDRIA AND ANÆMIA.

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As early as 1880, Samuel Fenwick in his observations on 'Atrophy of the Stomach' stated that 'the volume of blood depends on the quantity of nutriment dissolved and absorbed by the digestive tract'. The importance of failure of gastric function in the causation of anæmia was not realized until the methods for investigating it became more simplified. An analysis of gastric contents has now become a recognized laboratory investigation in the diagnosis of disease processes. Refinements in the technique of obtaining specimens for examination have made the procedure popular and analysis of gastric contents is frequently resorted to in the diagnosis of many morbid conditions. Over 600 analyses are carried out every year in the Central Clinical Laboratory of the King Edward VII Memorial Hospital, Bombay. Since such a large amount of material was available it was decided to study in greater detail cases exhibiting achlorhydria, a condition which is not infrequently met with in the cases sent for clinical investigation. During a period of seven months (1st September, 1937 to 30th April, 1938) 515 cases were investigated in the department and of these 102 showed achlorhydria.

The incidence of achlorhydria in general disease has been investigated by a large number of workers in countries having temperate climates and, combining both sexes, it appears to vary from 10 to 15 per cent, from observations of Andresen, Eggleston, Vanden Hoof, Hurst, Kohiyar, and others. Apperley on a basis of 200 cases gives a figure of 18 per cent and Nye and Sippe on an analysis of 350 cases give a figure of 21 per cent. We have gone through the fractional analysis of our cases for the last five years, 1933-1937 (inclusive), and on the results of 2,873 cases we find the percentage of cases showing achlorhydria as 20.8 per cent in agreement with the figures of the latter three Australian workers. It

has been suggested that the incidence of achlorhydria in the tropics is greater than in temperate climates and the result of our analysis seems to support this suggestion.

In recent years it has been realized that many cases of achlorhydria were not genuine cases of achlorhydria as they could be induced to secrete hydrochloric acid under certain conditions mainly after the use of gastric stimulants such as histamine. For various reasons it was possible to study only 75 cases by a repetition of the test analysis after histamine injection, and the results of these tests form the basis of this study.

The acid reaction of normal gastric secretion was demonstrated for the first time by Reaumur in the year 1752 and was later confirmed by Spallanzani. That it was hydrochloric acid which was responsible for the acidity was probably first demonstrated by Prout 80 years later. It took another 40 years or so before the device of using a tube for withdrawing the samples came to be introduced by von Leube in the year 1879. Later, Ewald, Boas and others employed a one-hour test-meal for the purpose of investigation. Gross suggested the use of soft catheter for taking out samples of gastric contents but the large-bore tube remained in use until the year 1920 when Einhorn, Rehfuß, Ryle, and others simplified the technique by the use of a fine-bore tube and demonstrated the value of a fractional test-meal analysis over the one-hour sample test after Ewald breakfast. Various other test-meals were introduced from time to time with a view to make the test more reliable at the same time simple. None of them, however, has been adopted so extensively as the gruel meal (oat meal) initiated by Rehfuß which is still employed in one of the standard methods of investigating gastric contents. Egglestone has shown that out of 100 patients showing absence of free HCl by one-hour test-meal method 50 showed hydrochloric acid when the fractional method was used. The cases taken up for investigation showed achlorhydria by the fractional method after the use of a gruel meal.

When a large number of gastric analyses came to be performed it soon became apparent that in some cases achlorhydria was present in the absence of any symptoms relating to the stomach or any general disease such as anæmia or any other obvious cause. In order to be able to interpret such findings it became necessary to find out the character of the gastric contents in healthy individuals. Bennett and Ryle investigated a series of 100 apparently healthy University students by the fractional method and found that four of them showed achlorhydria. Observations on an extended scale showed that the incidence of achlorhydria increased with increasing age. Hurst gives a figure of 10·3 per cent for ages between 20 and 80 years on the basis of 2,356 test-meals done on all patients admitted to the New Lodge Clinic, excluding cases of carcinoma, Addison's anæmia, and partial gastrectomy. Vanzant in her analyses of 3,381 cases with a normal digestive tract finds that the percentage of achlorhydria increased from 4 at the age of 20 years to 26 at the age of 60 years. N. Rao has done gastric analysis by the fractional method on healthy South Indians and gives the figure for achlorhydria as 7 per cent. Bhatia, Patel and Dundas (1931), working in Bombay, state that 27 per cent of normal healthy individuals showed achlorhydria; a figure higher than that of 20·8 per cent which we have found in general disease on an analysis of 2,873 cases. The number of cases on which they based their observations was only 30. We consider

this to be too small to work out a percentage. Further observations on the incidence of achlorhydria in normal healthy individuals would be necessary in order to obtain a correct idea of its incidence.

Criticism used to be made that the curve of acidity of a given subject may vary from day to day. Ryle (1926) mentions that there is no substantial variation in many of his cases and in himself when the fractional test-meal was repeated. He quotes Bell and Adam who repeated an analysis on 20 consecutive days on the same person and found that the general conformation of the curve was similar, although the curve obtained on the first day was distinctly lower than on subsequent days. It has been shown in some cases that the substitution of a meal different to the one previously given produces a different secretory response. In order to obviate this error Popielski in 1920 and Carnot, Koskawski and Leibert in 1922 suggested the use of histamine as a gastric stimulant. In the course of our work we repeated the analysis on every case of achlorhydria and it was found that on repetition free hydrochloric acid was found in the fasting sample of 11 cases of this group of 75. This clearly indicates that a single examination from the point of view of the diagnosis of achlorhydria cannot be relied on. Gompertz and Voorhaus in the year 1925 showed that a subcutaneous injection of histamine is useful in differentiating true achlorhydria in which the secreting glands have lost the capacity of producing hydrochloric acid from false achlorhydria in which the function of acid secretion was inhibited. In every case of achlorhydria by the fractional method where gastric analysis could be repeated we have tried the effect of histamine to determine whether the achlorhydria was false or true.

PROCEDURE.

The technique used was as follows:—

After an over-night fast the patient was asked to swallow the tube and the contents were aspirated. If the resting juice did not show the presence of free HCl in this second test the patient was given a subcutaneous injection of 0.5 c.c. of histamine acid phosphate (B.D.H.). A rise in pulse rate and a diffuse erythema were considered essential as showing that the substance was active. Three more specimens of juice were aspirated at an interval of 30, 45, and 60 minutes. The free and total acid present in them was estimated by the usual titration method. Although it has been mentioned that the pulse rate after the injection did not rise above 90, in some of our anæmic cases it was 90 per minute to start with and after the injection it rose to 120 to 130 per minute. In no case did any untoward effect such as headache, palpitation, or collapse manifest itself. Patients recovered readily from the slight flushing which was produced by the injection. In one case where a possibility of collapse was anticipated because of the extreme anæmia of the patient an injection of coramine was given soon after the injection of histamine and no unpleasant after effect followed. By this method out of a total of 75 cases of achlorhydria by the fractional method it was found that cases of true achlorhydria were only 39.

Wherever possible in all the 75 cases of achlorhydria taken up for study a detailed clinical history was taken and in addition to the gastric analysis

done in the manner described above, the following investigations were carried out:—

- (1) Cytological examination of blood, (2) urine examination, (3) sputum examination, (4) examination of fæces by ordinary and concentration method, (5) Kahn reaction on blood, (6) van den Bergh's reaction, (7) icterus index, and (8) in cases of suspected pellagra hæmatoporphyrin in urine.

Table I gives the provisional diagnosis of cases sent for investigation:—

TABLE I.

Anæmia	59
Sub-acute combined degeneration ..	4
Sprue	1
Leukæmia	1
Pellagra	5
Dyspepsia	2
Peptic ulcer	1
Cirrhosis of liver	1
Abdominal tuberculosis	1
Pulmonary tuberculosis	1
Cardiac failure	1
Cystitis	1

Table I shows that a large bulk of our cases were of people suffering from anæmia probably because it was in the diagnosis of the type of anæmia that a gastric analysis was often requisitioned. As a result of clinical and pathological investigations performed in the manner indicated above, although clinically only 59 cases were suspected to be anæmic, except for seven cases all the rest showed varying grades of anæmia, i.e., a hæmoglobin percentage below 71 which we have taken as an arbitrary standard. In two cases of paraplegia the investigations are incomplete

and in six cases, in spite of a careful investigation, it was not possible to arrive at any conclusion as regards their nature, and for this reason they were classified as 'undetermined'. Table II gives the classification of the anæmia cases:—

TABLE II.

True achlorhydria (39 cases).			False achlorhydria (36 cases).		
Hyperchromic anæmia	21	Pernicious anæmia .. 20 Ankylostoma .. 1	Hyperchromic anæmia	12	Tropical macrocytic anæmia .. 10 Ankylostoma .. 2
		Malaria .. 1 Syphilis .. 1 Cystitis .. 1			Malaria .. 2 Syphilis .. 1 Cystitis .. 1
Hypochromic anæmia	15	Ankylostoma .. 2 Witt's anæmia .. 4 Pellagra .. 4 Sub-acute combined degeneration .. 2	Hypochromic anæmia	19	Ankylostoma .. 4 Post-hæmorrhagic .. 2 Leukæmia .. 2 Paraplegia .. 2 Undetermined .. 5
No anæmia	3	Asthma .. 1 Dyspepsia .. 1 Pellagra .. 1	No anæmia	4	Malignancy liver .. 1 Dyspepsia .. 1 Pulmonary tuberculosis 1 Abdominal tuberculosis 1

The cases have been divided into two main groups, those that were cases of true achlorhydria and those that showed false achlorhydria. To the former belonged 39 cases, while 36 could be placed in the latter group. Generally speaking, therefore, it could be said that about half the cases which showed achlorhydria by the fractional method showed a true achlorhydria after histamine injection.

In Table II the cases have been further subdivided according as they showed a hyperchromic anæmia, hypochromic anæmia, or no anæmia. Below each of these divisions is given a list of the supposed ætiological factor responsible for that type of anæmia together with the number of cases that came under observation belonging to each type. Since the presence of achlorhydria (false) was the primary criterion for taking up the cases for study it was not surprising that there should be a preponderance of hyperchromic anæmias many of which are known to be of

nutritional origin and accompanied by a probable derangement of gastric function. Achlorhydria is only an indication that other secretions such as pepsin, rennin, and the intrinsic factor of the hæmatopoietic principle described by Castle may be absent from the gastric secretion. We have not made any observations on the presence or absence of the intrinsic factor but, regarding pepsin which was estimated by Fould's method, out of five cases it was found to be absent in four cases of true achlorhydria, and in one in which it was present its amount was less than normal. It might be mentioned in this connection that Davies (1930-31) has endeavoured to show that the failure of gastric function takes place gradually, the hydrochloric acid being the first to disappear and is followed by pepsin and finally by the intrinsic factor. This sequence of events does not always take place because cases have been described by Wilkinson and others in which intrinsic factor was found to be absent although hydrochloric acid was present. There is no doubt, however, that in true achlorhydria a larger number of cases show absence of pepsin and probably the intrinsic factor than in false achlorhydria. The absence of the intrinsic factor and the hæmatopoietic principle is believed to be responsible for the megaloblastic reaction of the bone-marrow and a hyperchromic type of blood picture. In the hyperchromic group more than half (20) belonged to the true Addisonian type of anæmia. For placing a case in this category we have relied, in addition to the hyperchromic nature of anæmia, on the clinical features and the presence of a true achlorhydria, i.e., complete absence of hydrochloric acid even after histamine stimulation. Presence of bilirubinæmia as shown by the icterus index, an increased indirect van den Bergh reaction and signs of sub-acute combined degeneration of the cord have been taken as additional considerations in support of the diagnosis of pernicious anæmia. Hurst (1932) has shown that pernicious anæmia is almost always associated with *achylia gastrica*. It has been mentioned that if free HCl is found present in the gastric contents of a suspected case of Addison's pernicious anæmia the diagnosis should be reconsidered though not of necessity altered. In a series of 209 cases Wilkinson found six with free acid only three of which were probably true cases of pernicious anæmia. Although pernicious anæmia can occur in the presence of free HCl we have thought it best to include only those cases which satisfy all the clinical and pathological requirements.

The next large group of our cases of the hyperchromic type have been placed under a group of what are called tropical megalocytic anæmias—a very unsatisfactory term. As a matter of fact all these cases would have appeared under the category of pernicious anæmia if reliance had been placed only on a fractional test-meal analysis. It is only histamine injection which has served to separate them out from genuine pernicious anæmia cases in the absence of an estimation of the intrinsic factor. Vaughan (1932) has given the following definition of this type of anæmia; 'A megalocytic hyperchromic anæmia with a normal indirect van den Bergh reaction occurring in tropical countries and not necessarily associated with diarrhœa, defective gastric secretion or pregnancy'. Except for one case in which the icterus index was 12 and therefore bilirubinæmia was probably present, the rest of the nine cases conformed to the definition given above. They showed a megalocytic hyperchromic blood picture and their indirect van den Bergh reactions and icterus indices were normal. They were not associated with pregnancy or diarrhœa and their gastric secretion showed achlorhydria by fractional test-meal method but

under histamine stimulation secreted hydrochloric acid. All other investigations were of a negative character.

Wills (1931) who has studied this type of anæmia extensively found it more common in females. Most of the cases described by her have been poor Mohammedan pregnant women, below thirty-five years of age, in whom the anæmia was relieved by administration of liver or alternatively with Marmite (yeast extract). It is believed that a deficiency of vitamin-B complex in the diet or a protein substance which serves as an extrinsic factor of the hæmatopoietic principle (supplied by Marmite) conduces to produce this hyperchromic macrocytic anæmia*. Seven out of 10 cases included in this group were males. Their average age was 37 years although extremes of 18 and 70 years were encountered. They belonged to three different religions, Hindu, Roman Catholic, and Mohammedan. The diversity of diet in people belonging to these three different groups is so great that it would be difficult to find out a substance which would be lacking in the diets of all these peoples. At any rate the subject requires further study.

Cases of ankylostomiasis were divided between the two main groups, hyperchromic and hypochromic—three belonging to the former and six to the latter. Three of these cases showed a true achlorhydria and in them the blood picture was of the hypochromic type. They showed large number of ankylostoma eggs in their stools. Four of these cases showed an increased icterus index, two of which presented a hyperchromic picture. In only two of them a well-marked eosinophilia was present, namely, 9 per cent and 15 per cent. In all others the eosinophile content of the blood was normal. It has been our experience from a study of large number of cases of ankylostomiasis that absence of eosinophilia is a common feature in undoubted cases of ankylostomiasis. As a matter of fact in one of these cases which showed *achylia gastrica* from the gastric contents ankylostoma worms could be recovered and in it the eosinophile content of the blood was only 3 per cent. In one of the cases of the hyperchromic type the icterus index was 20 and even after histamine stimulation the acidity was 8 per cent in terms of N/10 HCl. It is difficult to decide whether this may not have been a case which was on its way to a genuine Addison's pernicious anæmia and that ankylostoma infection was just a concomitant feature. The only point against this supposition was a 9 per cent eosinophilia which was seen in the blood picture.

In the group of hypochromic anæmia four cases belong to Witt's anæmia (simple achlorhydric). It is believed that this anæmia is more common in women. Two of our cases were males and two females. For separating this variety from other hypochromic anæmias complete absence of hydrochloric acid even after histamine injection and a characteristic spoon-shaped deformity of the nails were useful points.

This series includes five cases of pellagra clinically diagnosed as such. They did not show any marked anæmia. Four of them were males and one female. All of them showed a true achlorhydria. They showed typical skin lesions and one

* Since the preparation of this article for publication, Wills and Evans (1938) have suggested that the factor responsible for the production of tropical macrocytic anæmia is not vitamin B, but is probably contained in crude liver extract.

of them presented mental symptoms. The one test which was found to be useful in distinguishing these cases from allied conditions was the presence of hæmatoporphyrine in the urine. It was present in excess in every one of our five cases.

The rest of the cases which showed a true achlorhydria were of such varied ætiology that they included syphilis, malaria, cystitis, etc., and they do not call for any remarks except to note that the condition was probably accidental.

SUMMARY AND CONCLUSIONS.

The study of a series of cases of achlorhydria in Bombay has shown that:—

1. A single fractional test is not infallible from the point of view of the diagnosis of achlorhydria. On repetition free HCl was found in 11 cases out of a group of 75.
2. About half the number of cases which showed achlorhydria by the fractional test method secreted hydrochloric acid after histamine stimulation.
3. Where single analysis only is possible, a gastric analysis after histamine injection is advisable as this will serve to differentiate true from false achlorhydria.
4. The use of histamine is not attended with any risks even in severely anæmic cases.
5. Where true achlorhydria is found a large proportion of cases are likely to be cases of pernicious anæmia. Majority of other cases in this series were either Witt's anæmia, ankylostomiasis, or pellagra.

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* An extensive bibliography on the subject will be found in these papers from which details of some of the references in the article are cited.

PROTOCOLS.

PROTOCOL I.

Walt's anaemia. 4 cases.

Serial number.	Register number.	Age, years.	Sex.	Caste.	Histamine diagnosis.	Blood count.	van den Bergh's test.	Icterus index.	REMARKS.
1	K/3964	25	Female	Mohammedan	Achlorhydria	<div> <div>R.B.C., 3·3</div> <div>Hgb., 43 per cent</div> <div>C.I., 0·6</div> </div>	Spoon-shaped deformity of nails.
2	K/8806	24	Female	Hindu	Achlorhydria	<div> <div>R.B.C., 2·4</div> <div>Hgb., 28 per cent</div> <div>C.I., 0·6</div> </div>	Spoon-shaped nails.
3	L/888	32	Male	Hindu	Achlorhydria	<div> <div>R.B.C., 4·5</div> <div>Hgb., 64·2 per cent</div> <div>C.I., 0·7</div> </div>	Indirect positive.	7	Spoon-shaped nails.
4	K/12317	45	Male	Hindu	Achlorhydria	<div> <div>R.B.C., 1·5</div> <div>Hgb., 22·7 per cent</div> <div>C.I., 0·74</div> </div>	Indirect positive.	12	Spoon-shaped nails.

The figure in front of R.B.C. represents so many million red blood cells per c.mm.

PROTOCOL II.
Pellagra. 5 cases.

Serial number.	Register number.	Age, years.	Sex.	Caste.	Histamine diagnosis.	Blood count.	van den Bergh's test.	Icterus index.	REMARKS.
1	L/811 ..	50	Male	R. C.	Achlorhydia	R.B.C., 5.5 Hgb., 90 per cent Eos., 7 " " C.I., 0.9	Indirect positive.	7	Urinary porphyrin increased.
2	L/2566	23	Male	Hindu	Achlorhydia	R.B.C., 3.4 Hgb., 53.5 per cent Eos., 8 " " C.I., 0.86	Negative	1	Urinary porphyrin increased.
3	L/2277	50	Female	Hindu	Achlorhydia	R.B.C., 4.2 Hgb., 70.8 per cent C.I., 0.84	Negative	1	Urinary porphyrin increased; cord symptoms.
4	K/8976	27	Male	Hindu	Achlorhydia	R.B.C., 4.9 Hgb., 70.6 per cent C.I., 0.7	Negative	3	Urinary porphyrin increased; cord and mental symptoms.
5	K/1188	35	Male	Hindu	Achlorhydia	R.B.C., 4.0 Hgb., 40.2 per cent C.I., 0.5	Urinary porphyrin increased.

The figure in front of R.B.C. represents so many millions red blood cells per c.mm.

Protocol III.

Anamia ankylostomiasis. 9 cases.

(Stool examination showed plenty of eggs of ankylostoma.)

Serial number.	Register number.	Age, years.	Sex.	Caste.	Illistamino diagnosis.	Blood count.	van don Bergh's test.	Icterus index.	REMARKS.
1	K/11087	47	Male	Hindu	Achlorhydria	R.B.C., 4.0 Hgb., 50.2 per cent C.I., 0.03	Negative	3	..
2	K/11086	25	Female	Hindu	Achlorhydria	R.B.C., 1.2 Hgb., 26 per cent Eos., 6 " " C.I., 1.08
3	L/2927	45	Male	Hindu	Achlorhydria	R.B.C., 1.5 Hgb., 14.0 per cent Eos., 3 " " C.I., 0.5	Indirect positive.	7	Adult worms in gastric juice.

The figure in front of R.B.C. represents so many million red blood cells per c.mm.

PROTOCOL III—*concl.*

Serial number.	Register number.	Age, years.	Sex.	Caste.	Histamine diagnosis.	Blood count.	van den Bergh's test.	Icterus index.	REMARKS.
4	L/3009	22	Female	R. C.	Normal acidity.	R.B.C., 4.8 Hgb., 87.6 per cent Eos., 3 " " C.I., 0.9	Indirect positive.	10	..
5	L/3053	33	Male	R. C.	Normal acidity.	R.B.C., 2.6 Hgb., 44.3 per cent Eos., 15 " " C.I., 0.9	Negative	3	Kahn positive.
6	L/3078	40	Male	Hindu	Hypochlorhydria.	R.B.C., 1.4 Hgb., 30 per cent Eos., 9 " " C.I., 1.1	Indirect positive.	20	Large number of ova of round worms also detected.

7	L/3562	27	Male	Hindu	Normal acidity.	<div> <div>R.B.C., 1.4</div> <div>Hgb., 29 per cent</div> <div>Eos., 3 "</div> <div>C.I., 1.1</div> </div>	Indirect positive.	7	
8	L/1171	27	Male	Hindu	Hypochlor- hydria.	<div> <div>R.B.C., 1.4</div> <div>Hgb., 20 per cent</div> <div>C.I., 0.8</div> </div>	Negative	1	..
9	L/2222	25	Male	Hindu	Hypochlor- hydria.	<div> <div>R.B.C., 1.4</div> <div>Hgb., 21 per cent</div> <div>Eos., 5 "</div> <div>C.I., 0.8</div> </div>	Indirect	5	..

The figure in front of R.B.C. represents so many million red blood cells per c.mm.

PROTOCOL IV.

Tropical macrocytic anæmia. 10 cases.

Serial number.	Register number.	Age, years.	Sex.	Caste.	Histamine diagnosis.	Blood count.	van den Bergh's test.	Icterus index.	REMARKS.
1	K/5725	40	Male	Hindu	Normal acid-ity. {	R.B.C., 1.0 Hgb., 28 per cent C.I., 1.5
2	K/9374	40	Male	Hindu	Hypochlor- hydria. {	R.B.C., 1.4 Hgb., 35 per cent C.I., 1.3	Indirect positive.	5	..
3	K/9718	18	Male	Mohammedan	Hypochlor- hydria. {	R.B.C., 1.7 Hgb., 35.3 per cent C.I., 1.04	Negative	5	..
4	K/10415	37	Female	R. C.	Normal acid-ity. {	R.B.C., 2.9 Hgb., 67.2 per cent C.I., 1.16

5	K/1176	23	Male	Hindu	Normal acid-ity.	{ R.B.C., 1.8 Hgb., 36.3 per cent C.I., 1	}
6	L/3292	30	Female	Hindu	Normal acid-ity.	{ R.B.C., 2.5 Hgb., 55 per cent C.I., 1.08	} Indirect positive.	12	..
7	L/3071	37	Male	Hindu	Hypochlor-hydrin.	{ R.B.C., 2.1 Hgb., 44.9 per cent C.I., 1.2	} Indirect positive.
8	L/743	28	Female	Hindu	Hypochlor-hydrin.	{ R.B.C., 1.6 Hgb., 45.6 per cent C.I., 1.4	}
9	L/1497	70	Male	R. C.	Normal acid-ity.	{ R.B.C., 1.4 Hgb., 31 per cent C.I., 1.2	} Negative	3	Kahn positive.
10	L/2148	55	Male	Hindu	Normal acid-ity.	{ R.B.C., 2.6 Hgb., 65 per cent C.I., 1.2	} Negative	1	

The figure in front of R.B.C. represents so many million red blood cells per c.mm.

PROTOCOL V.

Sub-acute combined degeneration and pernicious anæmia. 22 cases.

Serial number.	Register number.	Age, years.	Sex.	Caste.	Histamine diagnosis.	Blood count.	van den Bergh's test.	Icterus index.	Diagnosis.
1	L/1631	25	Male	Hindu	Achlorhydia {	R.B.C., 2.2 Hgb., 44 per cent C.I., 1.1	Negative }	1	Sub-acute combined degeneration.
2	L/681	41	Male	Hindu	Achlorhydia {	R.B.C., 4.8 Hgb., 44 per cent C.I., 0.93	Negative }	1	Sub-acute combined degeneration.
3	L/349	60	Female	Hindu	Achlorhydia {	R.B.C., 2.9 Hgb., 52.8 per cent C.I., 0.91	.. }	..	Sub-acute combined degeneration.
4	L/1832	40	Female	Hindu	Achlorhydia {	R.B.C., 2.5 Hgb., 55 per cent C.I., 1.1	Indirect positive. }	5	Pernicious anæmia.

5	K/12029	30	Male	Mohammedan	Achlorhydria	<div> <div>R.B.C., 0.6</div> <div>Hgb., 14.9 per cent</div> <div>C.I., 1.2</div> </div>	Indirect positive.	5	Pernicious anaemia.
6	K/8907	45	Male	Hindu	Achlorhydria	<div> <div>R.B.C., 3.3</div> <div>Hgb., 72.7 per cent</div> <div>C.I., 1.1</div> <div>V.I., 1.2</div> </div>	Indirect positive.	5	Pernicious anaemia.
7	K/9633	33	Male	Hindu	Achlorhydria	<div> <div>R.B.C., 1.2</div> <div>Hgb., 28.8 per cent</div> <div>C.I., 1.3</div> <div>V.I., 1.4</div> </div>	Negative	3	Pernicious anaemia.
8	K/9862	26	Male	Hindu	Achlorhydria	<div> <div>R.B.C., 0.9</div> <div>Hgb., 24 per cent</div> <div>C.I., 1.4</div> </div>	Indirect	7	Pernicious anaemia.
9	K/9810	24	Male	Hindu	Achlorhydria	<div> <div>R.B.C., 1.8</div> <div>Hgb., 40 per cent</div> <div>C.I., 1.2</div> </div>	Pernicious anaemia.

The figure in front of R.B.C. represents so many million red blood cells per c.mm.

PROTOCOL V—concl'd.

Serial number.	Register number.	Age, years.	Sex.	Caste.	Histamine diagnosis.	Blood count.	van den Bergh's test.	Icterus index.	Diagnosis.
19	L/1423	40	Female	Mohammedan	Achlorhydria	R.B.C., 2.8 Hgb., 58.8 per cent C.I., 1.05	Negative	1	Pernicious anaemia.
20	L/1382	25	Male	Hindu	Achlorhydria	R.B.C., 2.6 Hgb., 54.5 per cent C.I., 1.1	Pernicious anaemia.
21	L/1400	53	Male	Hindu	Achlorhydria	R.B.C., 3.1 Hgb., 70 per cent C.I., 1.2	Indirect positive.	7	Pernicious anaemia.
22	K/11488	25	Male	Hindu	Achlorhydria	R.B.C., 0.7 Hgb., 23 per cent C.I., 1.6	Pernicious anaemia.

The figure in front of R.B.C. represents so many million red blood cells per c.mm.

PROTOCOL VI.

Miscellaneous. 25 cases.

Serial number.	Register number.	Age, years.	Sex.	Caste.	Histamine diagnosis.	Blood count.	van den Bergh's test.	Leucocyte index.	Diagnosis.	REMARKS.
1	L/1697	20	Female	Hindu	Hypochlorhydria.	R.B.C., 3.4 Hgb., 66 per cent C.I., 0.9	Negative	3	Undetermined	..
2	L/1695	20	Female	Hindu	Hypochlorhydria.	R.B.C., 2.7 Hgb., 28.8 per cent C.I., 0.53	Negative	1	Undetermined	..
3	K/12320	24	Male	Mohammedan	Normal acidity.	R.B.C., 3.56 Hgb., 39.5 per cent C.I., 0.56	Undetermined	..
4	L/1915	30	Female	Hindu	Hypochlorhydria.	R.B.C., 3.54 Hgb., 50.4 per cent C.I., 0.66	Indirect positive.	5	Myeloid leukæmia.	Myeloblasts, normoblasts, myelocytes 20 per cent.

The figure in front of R.B.C. represents so many million red blood cells per c.mm.

PROTOCOL VI—*contd.*

Register number.	Age, years.	Sex.	Caste.	Histamine diagnosis.	Blood count.	van den Bergh's test.	Interus index.	Diagnosis.	REMARKS.
L/1649	30	Male	R. C.	Normal acidity.	R.B.C., 2·7 Hgb., 52·4 per cent C.I., 0·96	Indirect positive.	7	Aluekæmic leukæmia.	Erythroblasts, lymphocytes 84 per cent, neutrophil myelocytes 12 per cent, erythroblasts 27 per cent, lymphoblast.
L/2066	35	Male	R. C.	Achylia gastrica.	R.B.C., 4·64 Hgb., 73 per cent Eos., 9 " " C.I., 0·88	Negative	3	Dyspepsia	..
L/4981	25	Male	Hindu	Normal acidity.	R.B.C., 5·52 Hgb., 90 per cent C.I., 0·9	Indirect positive.	7	Dyspepsia	..
L/2093	48	Male	Hindu	Hypochlorhydria.	R.B.C., 4·5 Hgb., 80·2 per cent C.I., 0·99 W.B.C., 15·0	Indirect positive.	7	Cystitis, and malignancy liver.	..

9	L/1852	?	Female	Hindu	<div> <div>Achlorhydria</div> <div> <div>R.B.C., 2·94</div> <div>Hgb., 54 per cent</div> <div>C.I., 0·93</div> <div>W.B.C., 12·35</div> </div> </div>	<div> <div>Negative</div> <div>}</div> </div>	1	Cystitis	..
10	K/10663	30	Male	Hindu	<div> <div>Normal acidity.</div> <div> <div>R.B.C., 1·97</div> <div>Hgb., 35 per cent</div> <div>C.I., 0·97</div> </div> </div>	<div> <div>..</div> <div>}</div> </div>	..	Cystitis	Urine—plenty of pus cells, albumin, Gram - negative bacilli.
11	L/2321	38	Male	Hindu	<div> <div>Normal acidity.</div> <div>..</div> </div>	<div> <div>Indirect positive.</div> <div>}</div> </div>	7	Pulmonary tuberculosis.	..
12	L/1378	30	Male	Hindu	<div> <div>Normal acidity.</div> <div> <div>R.B.C., 2·63</div> <div>Hgb., 56 per cent</div> <div>C.I., 0·5</div> </div> </div>	<div> <div>Indirect positive.</div> <div>}</div> </div>	5	Secondary anaemia due to piles.	..
13	K/12049	35	Male	Hindu	<div> <div>Hypochlorhydria.</div> <div> <div>R.B.C., 2·26</div> <div>Hgb., 37·4 per cent</div> <div>C.I., 0·9</div> </div> </div>	<div> <div>Negative</div> <div>}</div> </div>	1	Secondary anaemia due to malaria.	Spleen enlarged.
14	L/6	52	Male	Mohammedan	<div> <div>Achlorhydria</div> <div> <div>R.B.C., 3·06</div> <div>Hgb., 35·3 per cent</div> <div>C.I., 0·6</div> </div> </div>	<div> <div>..</div> <div>}</div> </div>	3	Secondary anaemia due to syphilis.	Kahn positive; normoblasts present.

The figure in front of R.B.C. represents so many million red blood cells per c.mm.

PROTOCOL VI—concl'd.

Serial number.	Register number.	Age, years.	Sex.	Caste.	Histamine diagnosis.	Blood count.	van den Bergh's test.	Icterus index.	Diagnosis.	REMARKS.
15	L/108	15	Male	Hindu	Achlorhydria	R.B.C., 1·7 Hgb., 17·1 per cent C.I., 0·5	Negative	3	Secondary anaemia due to malaria.	Spleen and liver enlarged.
16	K/79796	20	Female	Hindu	Normal acidity.	R.B.C., 4·3 Hgb., 84 per cent C.I., 0·98	Negative	3	Secondary anaemia.	..
17	K/10252	32	Male	Mohammedan	Normal acidity.	R.B.C., 2·4 Hgb., 25·6 per cent C.I., 0·6	Indirect	7	Secondary anaemia due to malaria.	..
18	K/9393	50	Male	Mohammedan	Hypochlorhydria.	R.B.C., 3·4 Hgb., 35 per cent C.I., 0·5	Negative	1	Secondary anaemia, undetermined.	..
19	K/11482	23	Female	Mohammedan	Hypochlorhydria.	R.B.C., 2·6 Hgb., 36 per cent C.I., 0·75	Anaemia due to P.P.H.	At the time of last confinement.

20	K/1173	35	Male	Hindu	Normal acidity.	{ R.B.C., 2.4 Hgb., 35 per cent C.I., 0.7	} Negative	3	Anæmia due to syphilis.	Kahn positive.
21	K/5463	25	Male	Mohammedan	Normal acidity.	{ R.B.C., 5.0 Hgb., 96 per cent C.I., 1	}	Paraplegia	..
22	K/9354	64	Female	Hindu	Hypochlorhydria.	{ R.B.C., 3.8 Hgb., 62.4 per cent C.I., 0.8	} Negative	5	Paraplegia	..
23	K/5128	26	Male	Mohammedan	Hypochlorhydria.	{ R.B.C., 1.0 Hgb., 16 per cent C.I., 0.9	}	Investigations incomplete.	..
24	K/8798	38	Male	Hindu	Achlorhydria	{ R.B.C., 17.500 Eos., 21 per cent	} Negative	3	Asthma	..
25	K/10537	30	Female	Parsee	Hypochlorhydria.	{	}	Abdominal tuberculosis.	..

The figure in front of R.B.C. represents so many million red blood cells per c.mm.

INVESTIGATION ON THE OCCURRENCE OF THE DANYSZ PHENOMENON IN DIPHTHERIA TOXIN-ANTITOXIN MIXTURES UNDER A VARIETY OF CONDITIONS.

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It was first observed by Danysz (1902) that a given quantity of ricin can combine with more antiricin when the toxin is added in two or more instalments with an interval of time between the successive additions, than when it is added in one instalment. This phenomenon has been further investigated by Danysz using diphtheria toxin and antitoxin, by Sachs (1904) using staphylolysin and antilysin and by Madsen and Arrhenius (1907) using tetanolysin and antilysin. Madsen and Arrhenius observed that the Danysz effect diminished with lapse of time and finally disappeared after six hours in the tetanolysin-antilysin mixture used by them. Recently, the Danysz effect in staphylococcus toxin-antitoxin mixtures has been investigated by us (Ghosh and Ray, 1937*a*) and also by Llewellyn Smith and Price (1938). We have found that the toxicity persists even twenty-four hours after the addition of the last fraction of the toxin and does not diminish appreciably. Several theories have been advanced to account for this phenomenon and these have been discussed in detail by Llewellyn Smith and Price (*loc. cit.*) and also by Ghosh and Ray (1937*a*). Schmidt (1928) records that using diphtheria toxin and antitoxin he could observe the Danysz phenomenon even in the flocculation reaction. Glenny (1931), however, points out that the occurrence of this phenomenon in the flocculation reaction is not to be expected from theoretical considerations. The present work was undertaken

with a view to ascertain (i) the conditions under which the Danysz phenomenon occurs in the flocculation of diphtheria toxin-antitoxin mixtures, (ii) the effect of prolonging the period of incubation of the toxin-antitoxin mixtures, after the addition of the last fraction of toxin, on the persistence of this phenomenon, and (iii) the effect of pH on its occurrence. The results so far obtained are recorded in this paper.

EXPERIMENTAL.

The Danysz effect in flocculation reaction.—Several samples of diphtheria toxin and antitoxin were selected for this purpose. In each set of experiments, three series of tubes were set up. In series I varying quantities of antitoxin were placed in the tubes and a fixed quantity of toxin was added in one instalment. In series II varying quantities of antitoxin were placed in the tubes as in series I, but the toxin was added in two instalments with a suitable interval of time. In series III a fixed quantity of toxin was placed in each of the tubes and varying quantities of antitoxin were added to each tube in two instalments with a suitable interval of time between the two additions. Each set of experiments was carried out in duplicate. After the addition of the toxin to the antitoxin or vice versa has been completed, the mixtures were kept at room temperature for about half an hour and then placed in a water-bath at 50°C. In the following tables K_f denotes the time required to produce just visible flocculation after the tubes have been placed in the hot water-bath. It will be noticed from the data recorded in Tables I to V that in any particular set of experiments the same mixture of toxin and antitoxin always flocculates first, independent of the mode of addition of the toxin to the antitoxin or vice versa. Thus, in Table I the mixture containing 1 c.c. of toxin and 0.7 c.c. of antitoxin always flocculates first. The value of K_f and the range of the mixtures in which flocculation occurs appear, however, to depend on the mode of addition of one substance to the other. The value of K_f is considerably increased when the antitoxin is added in two instalments to the toxin, while if the toxin is added to the antitoxin in two instalments the range of the mixtures in which flocculation occurs is markedly increased. In Tables I to III the interval of time between the two successive additions of toxin when the toxin is added fractionally is 15 minutes, while the corresponding interval of time in Tables IV and V is 30 minutes. In none of these experiments, however, could the Danysz effect be detected.

Effect of variation of the incubation period on the Danysz effect.—It follows from an analysis of the various mechanisms which have been suggested to account for the Danysz effect, that if the toxin-antitoxin reaction is reversible, then the Danysz effect should diminish and finally disappear as the interval of time (referred to above as incubation period) between the addition of the last fraction of toxin and the injection of the mixture to the animal is prolonged. Experiments were carried out to test this point. A sample of diphtheria antitoxin of known titre was diluted so as to contain one unit per c.c. Similarly, a sample of toxin was also diluted so that 1 c.c. of the dilute toxin mixed with 1 c.c. of the antitoxin and 1 c.c. of physiological saline and the whole mixture injected after an incubation of thirty minutes into guinea-pigs weighing 250 g. \pm 5, failed to kill the animals within 144 hours.

TABLE I.

Toxin No. RI₃.

Antitoxin No. 414.

Tube No. :—	1	2	3	4	5	6	7	8
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Series I.

Volume of antitoxin in c.c.	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
Volume of toxin in c.c.	1	1	1	1	1	1	1	1
K _f in minutes ..	—	—	—	—	105	45	135	—

Series II.

Volume of antitoxin in c.c.	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
Volume of toxin in c.c. added at 15 minutes' interval.	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
K _f in minutes ..	—	—	180	120	90	40	95	105

Series III.

Volume of toxin in c.c.	1	1	1	1	1	1	1	1
Volume of antitoxin in c.c. added at 15 minutes' interval.	0.1	0.15	0.2	0.25	0.3	0.35	0.4	0.45
	0.1	0.15	0.2	0.25	0.3	0.35	0.4	0.45
K _f in minutes ..	—	—	—	—	135	90	155	—

The sign '—' represents absence of flocculation.

TABLE II.

Toxin No. 55 B.

Antitoxin No. 371.

Tube No. :—	1	2	3	4	5	6	7	8
-------------	---	---	---	---	---	---	---	---

Series I.

Volume of antitoxin in c.c.	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
Volume of toxin in c.c.	1	1	1	1	1	1	1	1
K _f in minutes ..	—	—	180	110	150	240	—	—

Series II.

Volume of antitoxin in c.c.	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
Volume of toxin in c.c. added at 15 minutes' interval.	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
K _f in minutes ..	—	130	100	80	95	135	165	200

Series III.

Volume of toxin in c.c.	1	1	1	1	1	1	1	1
Volume of antitoxin in c.c. added at 15 minutes' interval.	0.15	0.2	0.25	0.3	0.35	0.4	0.45	0.5
	0.15	0.2	0.25	0.3	0.35	0.4	0.45	0.5
K _f in minutes ..	—	—	210	120	190	—	—	—

The sign '—' represents absence of flocculation.

TABLE III.

Toxin No. 52 B.

Antitoxin No. 306.

Tube No. :—	1	2	3	4	5	6	7	8	9
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Series I.

Volume of antitoxin in c.c.	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
Volume of toxin in c.c.	1	1	1	1	1	1	1	1	1
K _f in minutes ..	—	—	180	45	90	240	—	—	—

Series II.

Volume of antitoxin in c.c.	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
Volume of toxin in c.c. added at 15 minutes' interval.	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
K _f in minutes ..	180	150	110	40	60	90	125	188	—

Series III.

Volume of toxin in c.c.	1	1	1	1	1	1	1	1	1
Volume of antitoxin in c.c. added at 15 minutes' interval.	0.1	0.15	0.2	0.25	0.3	0.35	0.4	0.45	0.5
	1.1	0.15	0.2	0.25	0.3	0.35	0.4	0.45	0.5
K _f in minutes ..	—	—	260	60	180	—	—	—	—

The sign '—' represents absence of flocculation.

TABLE IV.

Toxin No. 31 B.

Antitoxin No. 413.

Tube No. :—	1	2	3	4	5	6	7	8
-------------	---	---	---	---	---	---	---	---

Series I.

Volume of antitoxin in c.c.	0.6	0.7	0.8	0.9	1.0	1.1	1.2	1.3
Volume of toxin in c.c.	2	2	2	2	2	2	2	2
K _f in minutes ..	—	—	—	110	72	120	—	—

Series II.

Volume of antitoxin in c.c.	0.6	0.7	0.8	0.9	1.0	1.1	1.2	1.3
Volume of toxin in c.c. added at 30 minutes' interval.	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
K _f in minutes ..	—	145	90	48	30	60	115	—

Series III.

Volume of toxin in c.c.	2	2	2	2	2	2	2	2
Volume of antitoxin in c.c. added at 30 minutes' interval.	0.3	0.35	0.4	0.45	0.5	0.55	0.6	0.65
	0.3	0.35	0.4	0.45	0.5	0.55	0.6	0.65
K _f in minutes ..	—	—	—	175	110	190	—	—

The sign ' — ' represents absence of flocculation.

TABLE V.

Concentrated toxin 1 c.c. contains 30 L_f units. [Freed from $(Ntl_4)_2SO$ by dialysis.]

Concentrated antitoxin diluted 30 times with normal saline. The diluted serum was used.

Tube No. :—	1	2	3	4	5	6	7	8
-------------	---	---	---	---	---	---	---	---

Series I.

Volume of antitoxin in c.c.	0.4	0.6	0.8	0.9	1.0	1.1	1.2	1.4
Volume of toxin in c.c.	2	2	2	2	2	2	2	2
K_f in minutes ..	—	—	—	180	100	220	—	—

Series II.

Volume of antitoxin in c.c.	0.4	0.6	0.8	0.9	1.0	1.1	1.2	1.4
Volume of toxin in c.c. added at 30 minutes' interval.	1.0 1.0	1.0 1.0	1.0 1.0	1.0 1.0	1.0 1.0	1.0 1.0	1.0 1.0	1.0 1.0
K_f in minutes ..	—	180	120	90	60	120	—	—

Series III.

Volume of toxin in c.c.	2	2	2	2	2	2	2	2
Volume of antitoxin in c.c. added at 30 minutes' interval.	0.2 0.2	0.3 0.3	0.4 0.4	0.45 0.45	0.5 0.5	0.55 0.55	0.6 0.6	0.7 0.7
K_f in minutes ..	—	—	—	300	180	—	—	—

The sign '—' represents absence of flocculation.

A series of tubes each containing 2 c.c. of the antitoxin and 2 c.c. of physiological saline were set up in groups of four and 2-c.c. quantities of the dilute toxin solution were added in two instalments at intervals of 15 minutes. After different periods of incubation 3 c.c. of the mixture were injected into guinea-pigs weighing 250 g. \pm 5. The results are recorded in Table VI. It will be noticed that the Danysz effect was strongly marked even 24 hours after the addition of the last fraction of the toxin.

TABLE VI.

Number of tubes set up.	Volume of antitoxin taken in c.c.	Volume of normal saline taken in c.c.	AMOUNT OF TOXIN ADDED IN C.C.		Period of incubation.	Number of animals used.	REMARKS.
			1st instalment.	2nd instalment.			
4	2	2	2	0	30 minutes	4	None died within 144 hours.
4	2	2	1	1	2 hours	4	All died within 48 hours.
4	2	2	1	1	4 "	4	All died within 48 hours.
4	2	2	1	1	8 "	4	All except one died within 48 hours.
4	2	2	1	1	24 "	4	All died within 48 hours.

Effect of hydrogen-ion concentration on the occurrence of the Danysz phenomenon.—

It has been observed by Schmidt (1930) and also by Ghosh and Ray (1937b) that the flocculation reaction is influenced by the hydrogen-ion concentration of the solution. It is, however, not yet known whether the pH of the medium has any effect on the occurrence of the Danysz phenomenon. To ascertain this point some experiments were undertaken. The pH of the toxin and antitoxin solutions was adjusted at the requisite value. They were then diluted with physiological saline also adjusted at the desired pH, so that 1 c.c. of the dilute toxin mixed with 2 c.c. of the dilute antitoxin and the whole mixture after 30 minutes' incubation injected into guinea-pigs of proper weight (250 g. \pm 5) failed to kill the animals within 120 hours. A series of tubes, in groups of four, were set up and 4 c.c. of the dilute antitoxin were added to each of them. The results are recorded in Table VII. In those experiments in which the toxin was added in two portions, an interval of 15 minutes

was allowed between the two additions. Three c.c. of the toxin-antitoxin mixtures were injected into guinea-pigs 45 minutes after addition of the last portion of toxin. The Danysz phenomenon was quite marked between pH 4.2 and pH 9.6.

TABLE VII.

pH of the solution.	Volume of antitoxin taken in c.c.	VOLUME OF TOXIN ADDED IN C.C.		Number of animals injected.	REMARKS.
		1st instalment.	2nd instalment.		
4.2	4	2	0	4	None died within 144 hours.
	4	1	1	4	All died between 48 and 96 hours.
6.0	4	2	0	4	None died within 144 hours.
	4	1	1	4	All died within 96 hours.
8.0	4	2	0	4	None died within 120 hours.
	4	1	1	4	All died within 96 hours.
9.6	4	2	0	4	None died within 120 hours.
	4	1	1	4	All except one died within 48 hours.

CONCLUSION.

1. The alleged occurrence of the Danysz phenomenon in the flocculation reaction has been investigated using several samples of diphtheria toxin and antitoxin. In one set of experiments the interval of time between the addition of the first and second fraction of toxin was 15 minutes, in another set of experiments the time interval was 30 minutes. In none of these experiments could the occurrence of the Danysz effect be detected.

2. The Danysz effect in diphtheria toxin-antitoxin mixture determined by tests on animals was strongly noticeable even 24 hours after the addition of the last fraction of toxin.

3. The Danysz effect in diphtheria toxin-antitoxin mixture is quite marked within the pH range 4.2 to 9.6.

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PHYSICO-CHEMICAL CHANGES IN A THERAPEUTIC SERUM DUE TO CONCENTRATION AND THEIR EFFECT ON ITS ABSORPTION.

BY

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THE adoption of International standards for several therapeutic sera by the League of Nations' Health Organization and improvements in the technique of standardization, have naturally led to their recognition as suitable therapeutic substances with accurately measurable dosage. It is now customary to state the content of therapeutic substances in a supply of serum in terms of International units per unit or total volume which is supposed to indicate its relative therapeutic efficacy.

The same procedure is applied both in cases of natural and concentrated sera. It is apparently assumed that there is no difference in value between a natural and a concentrated serum, excepting that the latter contains the requisite therapeutic substances in a smaller volume of serum and is free from those undesirable proteins which produce unwanted complications. Thus, a concentrated serum seems to possess distinct advantages over a natural one. Is this impression justified?

In the process of concentration the natural serum is treated with moderate severity. It is true that the treatment does not affect the property of the so-called pseudo-globulin fraction (that part of it which is not removed by the method of concentration employed) of an antiserum of combining specifically with the corresponding antigen. But do the changes in the physico-chemical properties resulting from concentration, produce no change at all in the therapeutic efficacy of a serum?

The efficacy of a drug, which acts when absorbed in the general circulation, may be affected by changes in its absorbability. This is especially true in the case of therapeutic sera which are employed for a rapid control of a disease and which are more frequently administered through routes other than intravenous. Do the various physico-chemical changes in a serum effected by processes of concentration then affect its absorption to any extent?

The present work is intended to show the effect of concentration of a diphtheria antitoxic serum on its absorbability, assuming that the conclusions derived from the results obtained in this case would be applicable to all cases of therapeutic sera and antitoxic sera in particular.

PHYSICO-CHEMICAL DETERMINATIONS.

Only some of the physico-chemical conditions considered to be important from the standpoint of their effect on absorption of sera were determined.

Several natural and concentrated sera were chosen at random. Of these, three samples of concentrated sera were obtained from the market. Each sample was divided into two portions. One was used for the determination of viscosity, etc., and the other stored at 0°C. to 2°C. for absorption experiments.

Relative viscosity was determined with Ostwald viscosimeter, surface tension with Traube stalagmometer and protein concentrations with Zeiss dipping refractometer (Siebenmann, 1937). Results are shown in Table I.

Thus, all the concentrated sera tested, with the exception of 'Foreign B', are found to be highly viscous and their protein concentrations increased, in comparison with the natural sera tested. Viscosity and protein concentration of 'Foreign B', though much lesser than those of other concentrated sera, are also higher in comparison to the natural sera. Differences in surface tension are relatively insignificant and so also are differences in pH.

RELATIVE RATES OF ABSORPTION OF NATURAL AND CONCENTRATED SERA.

Methods.

Determination of the antibody titre of sera used for absorption tests.—The particular lot of diphtheria toxin used for titration of all sera by the flocculation method, as done in these experiments, was more than one year old, preserved with 0.5 per cent phenol and had an L_f dose of 1/12 c.c. when titrated with standard antitoxin. The serum to be tested is first diluted according to its expected antibody titre, for instance, in cases of natural sera 1/20 or 1/30 and in cases of concentrated sera 1/100 or 1/200, using a tuberculin syringe for the measurements of volume. In a series of one-third inch test-tubes of uniform bore, 0.6 c.c., 0.65 c.c., 0.7 c.c., 0.75 c.c., 0.8 c.c., 0.85 c.c., 0.9 c.c., 0.95 c.c., and 1.0 c.c. of the diluted serum are accurately measured in with a tuberculin syringe. The volume in each tube is made up to 1.0 c.c. with saline, using the same syringe washed several times with saline. One c.c. of the toxin is put in each tube, also measured with tuberculin syringe. The contents in each tube are mixed up with a Pasteur pipette, beginning from tubes containing lower strength of serum and gradually proceeding to those containing higher strength. The tubes are put in a rack in a water-bath with glass walls and mechanism for oblique illumination so that flocculation can be observed while the tubes are in the bath, thus eliminating any possible error due to fluctuations of temperature while bringing the tubes out of the bath to take readings. The level of water in the bath is so adjusted that about two-thirds of the mixture in each tube remain inside water-level and one-third outside it, to allow of the continuous agitation of the mixtures with convection

current. The temperature of the bath is adjusted at 50°C. A fairly accurate result is obtained with this technique. As, for instance, to take a concrete case, 0.8 c.c. of 1:20 dilution of a natural serum showed earliest flocculation with 1 c.c. of toxin (L_f 1:12 c.c.). According to calculations, the antibody titre was 300 A. U. per c.c. Had the earliest flocculation occurred with 0.75 c.c. or 0.85 c.c. of the serum dilution, the titre would have been 320 A. U. or 282 A. U., respectively. So the range of possible error (± 20 A. U. in this case) is very narrow. In cases of titration of concentrated sera, the range appears to be little wider. To take a concrete case for instance, 0.75 c.c. of a 1:200 dilution of a concentrated serum showed earliest flocculation with 1 c.c. of toxin (L_f 1:12 c.c.). Hence the calculated antibody titre was 3,200 A. U. per c.c. In case of earliest flocculation with 0.7 c.c. or 0.8 c.c. the titre would have been 3,428 A. U. or 3,000 A. U., respectively. So the range of possible error was ± 200 A. U. But when compared with the high titre of these sera, the error is found to be of the same magnitude as that in cases of titration of natural sera.

Routes of injection.—Three routes, subcutaneous, intramuscular, and intravenous, are selected for the injection of diphtheria antitoxic sera. Sera injected by the subcutaneous and intramuscular routes only require to be absorbed. Whereas, the intravenous route is meant for introducing the sera directly in the circulation and in these experiments serves as a control measure of the degree of dilution of antibodies by the total volume of blood of a rabbit.

Collection of samples of blood.—Rabbits are bled from the heart with a 10-c.c. Record syringe having a wide-bored needle. The amount to be drawn depends on the range of titre expected. But when it is decided, the same amount is drawn from all the rabbits in a test, as an attempt to equalize in all the rabbits the effect of bleeding on the further rate of absorption of sera. Rabbits stand these bleedings quite well, as was seen from the survival of all the rabbits used for the test with market sera, if the operation is done carefully. The samples are stored at 0°C. to 2°C. after separating the clot from the wall of the test-tubes.

Titration of serum from the samples.—The serum collected from each sample is centrifuged to free it from red blood corpuscles. Different volumes are then measured in tubes already described, according to the range of titre expected. Amount of toxin used for these experiments is 0.5 c.c. excepting in cases where a titre of 3 A. U. per c.c. is intended to be detected, where 0.25 c.c. of toxin (L_f 1:12 c.c.) is mixed with 1 c.c. of rabbits' serum. The usual gradation of the volumes of a serum and the titre of antitoxin each one is intended to determine is as follows:—

Undiluted serum 1.0 c.c. + 0.25 c.c. toxin = 3 A. U. 0.5 c.c. of toxin is used for the rest of the series, where flocculation with 1.0 c.c. = 6 A. U.; with 0.665 c.c. = 9 A. U.; with 0.5 c.c. = 12 A. U.; and with 0.4 c.c. = 15 A. U.

Serum diluted 1:2 — 0.7 c.c. = 17 A. U.; 0.6 c.c. = 20 A. U.; 0.48 c.c. = 25 A. U.; 0.4 c.c. = 30 A. U.

Serum diluted 1:4 — 0.68 c.c. = 35 A. U.; 0.6 c.c. = 40 A. U.; 0.53 c.c. = 45 A. U.; 0.48 c.c. = 50 A. U.; 0.44 c.c. = 55 A. U.; 0.4 c.c. = 60 A. U.

Serum diluted 1:8 — 0.74 c.c. = 65 A. U.; 0.68 c.c. = 70 A. U.

Thus, it is seen that up to 20 A. U. a difference in titre of 3 A. U. and in the rest of the series a difference of 5 A. U. only is detected. This appears to be a determination of only gross differences in titre between any two samples. But considering the nature of various sorts of errors liable to creep in these sorts of experiments with different animals, a difference in titre less than that is actually determined, would appear to be of no significance. It is to be noted also that a titre recorded as 12 A. U., for instance, might be anything between 9 A. U. and 15 A. U. and that recorded as 30 A. U., for instance, might be anything between 25 A. U. and 35 A. U. The consideration of these possibilities render the accurate interpretation of the results difficult.

All these volumes are measured accurately with a tuberculin syringe. The volume in each tube is then made up to 10 c.c. with saline, using the same syringe. The toxin is added, mixed with Pasteur pipette and the tubes are then put in the bath described before. The total volume of mixture in each tube is 1.5 c.c. excepting where 0.25 c.c. of toxin is added, when the volume is 1.25 c.c. These differences in volume do not cause any fallacious reading.

Experimental.

1. *Relative rates of absorption of natural and concentrated sera by different routes.*—Two natural and two concentrated sera were selected at random and their physico-chemical conditions determined (Table I). Each sample was injected subcutaneously, intramuscularly, and intravenously in three different rabbits. The dose for subcutaneous and intramuscular injections was 6,000 A. U. per kg. body-weight. The dose for intravenous injection was 2,000 A. U. per kg. body-weight. The time of injecting each rabbit was noted. Seven c.c. of blood were drawn from each rabbit 3 hours and 24 hours after the injection. The samples were stored at 0°C. to 2°C. after separating the clot. The amount of serum available from each sample was from 3 c.c. to 3.5 c.c. Samples of any particular period were titrated all at one time, so as to ensure uniform storage period for all the samples to be compared. The range of dilutions made from each sample varied according to the route of injection, the time of collection of samples and the amount of serum available for making those dilutions. But for samples collected at the same time interval from rabbits injected by the same route irrespective of the nature of serum injected the same range of dilutions was made, for a fair comparison of the antibody titre in each sample. In one or two cases the earliest flocculation took place at one end of the range instead of in the middle. These happened in mixtures intended to read an antibody titre of 3 A. U. or 6 A. U. But from the rapidity of flocculation and failure of flocculation in the adjacent mixture for a fairly long time, it was taken that those were in all probability the optimal proportion mixtures.

The results of the experiment are shown in Table II.

The results signify that the highest titre of the circulating antibodies is obtained almost immediately after intravenous injection of a serum and it falls moderately by the 24th hour; whereas after subcutaneous or intramuscular injection the titre goes on increasing during the 24 hours of study.

Hence, the ratio of total volumes absorbed = 10.7 (approximate), assuming that the effects of dilution of antibodies by the total volume blood of the respective rabbits are identical.

Therefore 'Natural 1' serum was absorbed 10.7 times more than 'Concentrated 1' serum. But at the same time a great difference between the volume of the two sera injected was noted. Hence to assess properly the degree of effect of concentration on slowing the rate of absorption, it was necessary to find out the extent to which volume injected determined the amount absorbed in a given time.

2. *Relation of the volume of serum injected to the amount of antibody absorbed.*—Two samples of natural sera with different antibody titres were selected. Both of them were injected intramuscularly in a dose of 6,000 A. U. per kg. body-weight into two rabbits for each sample. Five c.c. of blood were withdrawn from each rabbit at the ends of the 6th and the 24th hours of injection and titrated as before.

The results are shown in Table III:—

TABLE III.

Relation of the volume of an antiserum injected with the amount absorbed in a given time.

Serum lot.	Units per c.c.	Relative viscosity.	Weight of rabbit, g.	Volume injected, c.c.	AMOUNT ABSORBED ; IN UNITS PER C.C.	
					6 hours.	24 hours.
'Natural 4' .. {	400	1.84	990	14.85	20	55
	400	1.84	1,010	15.15	12	40
'Natural 5' .. {	210	1.90	945	27	15	35
	210	1.90	915	26	12	40

To compare the ratio of the rates of absorption to that of the volumes injected of 'Natural 5,' to 'Natural 4', we get,

mean of the amount of 'Natural 5' absorbed per c.c. rabbits' serum
in 6 hours. = $\frac{13.5}{210}$ c.c.

whereas, " " " " 'Natural 4' absorbed per c.c. rabbits' serum
in 6 hours. = $\frac{16}{400}$ c.c.

Hence, ratio of the amounts absorbed = 1.61 (approximate).

Whereas, " " mean volumes injected = 1.77 (approximate).

Thus, it appears that, other conditions being equal, the amount absorbed is fairly proportional to the volume injected, though it is to be emphasized that these arithmetical calculations are never intended to represent the accurate state of relationship between the volume injected and the amount absorbed.

To revert to the consideration of relative rates of absorption of 'Natural 1' and 'Concentrated 1' sera in the previous experiment, the ratio of the volume of 'Natural 1' to that of 'Concentrated 1' injected = 5.1. Whereas, the ratio of their respective amounts absorbed = 10.7. Hence, the diminished rate of absorption of 'Concentrated 1' cannot be fully explained by the difference in volume alone. It appears that some sort of change in its constitution, effected by the process of concentration, must have been responsible for its diminished absorbability.

The total volume of rabbits' blood diluting a serum absorbed also deserves consideration for these calculations. But assuming that the volume of blood is proportional to the body-weight of an animal, the effect of this dilution on the relative antibody titre per c.c. of blood in different animals should be equalized by calculating the amount of antibodies to be injected in animal on the basis of its body-weight. Thus, a comparatively lower amount of antibody absorbed in an animal of lower body-weight, is compensated by a lower degree of dilution by a lower volume of blood proportional to its lower body-weight. That these assumptions hold good in practice is shown by the equal antibody titre in the samples from different rabbits injected intravenously with natural and concentrated sera, whose dosages were proportional to their body-weights.

3. *Relative rates of absorption of several concentrated sera in the market.*—Naturally, the question arises whether all these physico-chemical changes occurring in a serum due to its concentration and their effect on its absorption, is peculiar only to those prepared in a particular laboratory with particular methods, or they are general. The best answer to this question could possibly be given by testing several sera of different manufacture, both local and foreign, obtained from the market where only those sera, considered to be possessing the best possible therapeutic efficacy in respect of their drug content, are expected to be found.

Three samples—one local and two foreign—were bought from the market. A portion of each sample was used for physico-chemical determinations, while the other was used for testing its absorbability. The results of physico-chemical determinations are shown in Table I.

For the determination of their relative rates of absorption a natural serum was also included in the tests with those three samples. Eight rabbits—two for each sample—were selected. The dose selected was 4,200 A. U. per kg. of body-weight; injections were given intramuscularly. Samples of blood were drawn at the ends of the 5th, 8th, 26th, 50th, 100th, and 150th hours. Seven c.c. of blood were drawn for the first and the last samples, and 5 c.c. for the rest of the samples.

The results are shown in Table IV:—

TABLE IV.

Relative rates of absorption and elimination of several concentrated sera in the market and a natural serum, injected intramuscularly.

Serum lot.	Units per c.c.	Weight of rabbit, g.	Volume injected, c.c.	RATE OF ABSORPTION AND ELIMINATION IN UNITS PER C.C. OF RABBIT SERUM.					
				5 hours.	8 hours.	26 hours.	50 hours.	100 hours.	150 hours.
'Local B'	1,400	1,410	4.3	6	12	35	35	20	6
	1,400	1,460	4.4	6	15	35	30	12	3
'Foreign P'	3,200	1,425	1.9	3	9	20	35	25	17
	3,200	1,480	1.95	6	12	35	30	20	6
'Foreign B'	2,650	1,395	2.2	9	17	45	40	20	6
	2,650	1,440	2.3	6	15	30	40	30	12
'Natural 3'	440	1,480	14.1	9	17	50	40	20	6
	440	1,510	14.4	12	20	50	50	25	9

Hence, 'Local B' and 'Foreign P', both of which have very high viscosities, are absorbed to a much lesser extent than the other two. The antibody titres in sera of rabbits injected with these two are very low from the beginning and only approach to equality with the others when the rate of elimination far exceeds the rate of absorption.

The rate of absorption of 'Foreign B' also appears to be slightly less than that of 'Natural 3', which corresponds to only a slight difference in viscosities between those two.

It is also noticeable from the results of the experiment that in some rabbits the antibody titres in initial samples are lower than those of another rabbit injected with the same serum. But the titres in later samples are higher than those of another rabbit, showing that slow absorption and consequent slow elimination of antibodies in that particular rabbit, perhaps due to some anatomical or other sort of variation, is responsible for this apparent discrepancy.

The results of the experiment show that the physico-chemical changes occurring in a serum due to its concentration and their effect on its absorption is not peculiar to sera of a particular manufacture, but is general.

DISCUSSION.

Protein molecules cannot be absorbed into the circulation by a process of osmosis and diffusion through the semi-permeable capillary walls, due to their large size.

It is generally believed that they enter the circulation or leave it by a process of ultra-filtration through the capillary walls. Ultra-filtration implies passage of the filtrant through a number of capillary tubes of the same or varying length and diameter, depending on the nature of the filter. According to Hagen-Poiseuille's law of capillary flow,

$$m = \frac{\pi Pr^4 t}{8 \eta l}$$

where m = amount of fluid passing in time t through a capillary tube of radius r and length l under a hydrostatic pressure P ; η being the viscosity of the filtrant.

If it is assumed that the particular type of filter under consideration—the capillary wall—consists of capillary spaces of the same radius and length under normal conditions, and contains the same number of such spaces per unit of filtering surface, then

$$\frac{m_1}{m_2} = \frac{P_1 S_1 \eta_2}{P_2 S_2 \eta_1}$$

where m_1 and m_2 are the respective amounts of two filtrants filtering in a given time through the respective filtering surfaces S_1 and S_2 under respective hydrostatic pressures P_1 and P_2 , where η_1 and η_2 are the respective viscosities of the two filtrants—provided that the walls of such capillary spaces possess no electro-chemical affinity for the particles in the filtrant.

The hydrostatic pressure (P_1, P_2) and the area of filtering surface (S_1, S_2) are determined to a great extent by the volume of the fluid injected through a given route. Injection causes stretching of the muscular fibres when given intramuscularly and of the elastic tissue of the skin when given subcutaneously, the degree of stretching depending on the volume of fluid injected. The natural tonicity of those tissues exert pressure on the fluid injected, the amount of pressure depending on the degree of stretching on those tissues and thus on the volume of fluid injected. Also the area through which a fluid is spread after injection, or, in other words, the extent of capillary surface availed of for filtration, depends on the volume of fluid injected. Hence,

$$\frac{m_1}{m_2} = \frac{V_1 \eta_2}{V_2 \eta_1}$$

where V_1 and V_2 represent the respective volumes of fluid injected through a given route. It is understood that the effect of volume on the pressure exerted by a tissue and the area of capillary surface available in a tissue for filtration depends greatly on the anatomical nature of a tissue. Hence, this equation is valued only for comparing the relative amounts filtering through the capillary wall after injection of fluids of different volumes and viscosities into tissues of the same anatomical nature.

The assumptions that have been made in order to establish the equation governing the laws of filtration through the capillary wall, a living biological filter, are perhaps too ideal to be true and hence it is possibly too much to expect the relationship stated in the equation to be exact. But that this relationship is true, at least qualitatively, is shown by the results of the experiments, which clearly

indicate the importance of viscosity and volume of a serum injected in determining the amount of antibody particles filtering through the capillary wall.

In applying the equation to the results of the present experiments considerations should also be given to the technical difficulties. The results of the toxin-antitoxin titrations, which are the basis for determination of the amounts filtering through, are only approximate. Rabbits used in the experiments are likely to differ from one another anatomically in relation to the sites of injection and also as a biological unit in respect of its behaviour towards the serum injected. These differences in structure and constitution among the rabbits add to the difficulty in proper interpretation of the mode of action of the filter, the capillary wall, contained in them.

However, coming to the practical aspect of the proposition, the conclusion is that a concentrated serum which is more viscous is likely to be absorbed less rapidly and hence possibly has lesser therapeutic efficacy, in comparison with a natural serum of lesser viscosity, when both are injected in the same dosage depending on their antibody content. The consideration of volume factor appears to be unimportant for practical purposes, as from experimental evidence (Table III) it is clear that the antibody titre in serum of rabbits injected with a small volume of high-titre serum is about equal to that in rabbits injected with a large volume of low-titre serum, if the antibody content of two sera irrespective of the volume and their viscosities are equal.

The concentrated antidiphtheritic sera in the market, which have been experimented with, are more viscous than a natural serum and all of them are absorbed more slowly and in proportion to their viscosities, in comparison with a natural serum (Table IV).

Concentration of a serum to increase its antibody titre will increase its total protein content until it will be possible to eliminate most of the proteins in a serum with the least loss to the antibody molecules per unit of volume, when a contraction in volume would increase the antibody titre with very little rise in protein concentration. The evidences collected till the present time (*see* Marrack, 1938a), which show that the fraction of the globulin which is intended to be retained in a concentrated serum after the elimination of other fractions is greatly increased on immunization in most of the cases, do not suggest that this is possible at present. However, the antibodies in certain horse sera can be separated, highly purified, in a fraction of serum globulin (Marrack, 1938b). In this connection the observations of Tiselius and Kabat (1938) appear to be significant. They have shown by electrophoretic analysis of potent type I anti-pneumococcus horse sera that the antibody produced by the horse exists as a new globulin component in addition to the normal α -, β -, and γ -globulin components. Perhaps the separation of this component by the processes used gives such good results. But it does not appear to be very successful with other antibodies and antitoxins in particular.

As, according to Einstein's equation based on kinetics of the colloid particles in a solvent and other modifications of the original equation (Pauli and Valkó, 1933), viscosity of a solvent depends on the concentration of solute, it appears that increased concentration of colloidal protein particles in a concentrated serum is at least partly responsible for its high viscosity. It is true that such factors as

changes in the type and content of electrolytes, degree of dissociation of proteins, its hydration and swelling might all be responsible for determining the final viscosity. But in the present experiments, only the effect of increased protein concentration in increasing the viscosity of a concentrated serum is clearly demonstrated (Table I).

Hence concentration of a serum to increase its antibody titre, also increases in most of the cases its protein concentration, which causes a rise in viscosity of a serum. From theoretical considerations supported by experimental evidences, it appears that a rise in viscosity of a serum retards its absorption into the circulation and so possibly causes a diminution of therapeutic efficacy of a serum. Thus, the chain of inter-relationship of the factors modifying the therapeutic efficacy of a concentrated serum, appears to be complete.

SUMMARY.

Physico-chemical determinations show that the concentrated antidiphtheritic sera tested in the experiments are more viscous than the natural sera and the protein concentrations of the former are higher than those of the latter. Experiments show that the rates of absorption of the concentrated sera are lesser than those of the natural sera, when both of them are injected in rabbits in the same dose of units of antitoxin per kg. of body-weight.

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INVESTIGATION ON THE ISOLATION OF THE ACTIVE PRINCIPLES FROM THE VENOMS OF *BUNGARUS FASCIATUS* AND *VIPERA RUSSELLII*.

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It is generally believed that the active principles constitute a very small fraction of the total amount of the crude venom extracted from a poisonous snake. This view has been confirmed by the results which have recently been obtained by Ganguly and Malkana (1936a), by Ghosh and De (1938), and by Slotta and Fraenkel-Conrat (1938). Ghosh and De (*loc. cit.*) have shown that the neurotoxin of the cobra (*Naja Naja*) venom can be concentrated in a protein fraction which constitutes only one-sixteenth of the total protein of the crude venom. Slotta and Fraenkel-Conrat (*loc. cit.*) report that they have succeeded in separating the hæmolysin and neurotoxin of the *Crotalus terrificus* venom in a crystalline form constituting about 60 per cent of the crude venom. Since it has not yet been possible to obtain the active principles of the venoms of banded krait (*B. fasciatus*) and Russell's viper in a purified form experiments were undertaken to explore the possibility of their isolation. The results so far obtained are recorded in this paper.

SEPARATION OF THE ACTIVE PRINCIPLES FROM THE CRUDE RUSSELL'S VIPER VENOM.

(i) *First stage of removal of the inert proteins.*—The venom of the Russell's viper contains three active principles; a neurotoxin, a hæmorrhagin, and a blood coagulating agent. Ganguly and Malkana (1936b) succeeded in removing 50 per cent of the inactive proteins by fractional precipitation of a solution of Russell's

viper venom by ammonium sulphate. In our experiments we preferred to use anhydrous sodium sulphate as the protein precipitating agent for reasons stated in our previous paper (Ghosh and De, *loc. cit.*). To each of a number of conical flasks was added 20 c.c. of a 0.5 per cent solution (pH 5.8) of Russell's viper venom. The M. L. D. of the venom for pigeons weighing 300 g. to 310 g. was found to be 0.01 mg. Calculated quantities of a 40 per cent solution of sodium sulphate were added to the different flasks so that the final concentration of the sodium salt in them was 22, 23, 24, and 25 per cent, respectively. The flasks were then kept for half an hour in a thermostat at 37°C. and after that their contents were centrifuged separately. The protein and toxicity of the precipitate were then determined. The results are recorded in Table I. It will be noticed that as the concentration of sodium sulphate is increased the percentage of protein precipitated and the toxicity removed with it also increase. In all later experiments we precipitated the first fraction of proteins at 24 per cent concentration of sodium sulphate.

TABLE I.

Concentration of sodium sulphate in per cent.	Percentage of protein precipitated.	Percentage of neurotoxin precipitated.
22	24.1	5.5
23	33.6	8.0
24	39.2	10.0
25	42.3	18.0

(ii) *Second stage of removal of proteins.*—To the supernatant solution obtained after removing part of the proteins at 24 per cent concentration of sodium sulphate solution, was added solid sodium sulphate until its concentration reached 30 per cent. It had been found in preliminary experiments that precipitation at this concentration of sodium sulphate gives the most satisfactory results. The precipitate (ii) contains 40 per cent of the protein and 82 per cent of the neurotoxin of the crude venom used.

(iii) *Third stage of removal of proteins.*—The precipitate (ii) obtained at 30 per cent sodium sulphate concentration was dissolved in water and the adsorption of the protein and the toxins from this solution by a number of adsorbents were studied. The results are recorded in Table II. In these experiments the volume of the mixture was in each case 30 c.c. and it contained 50,000 M. L. D. of neurotoxin. It will be noticed that (Willstätter's) aluminium hydroxide C adsorbs relatively more neurotoxin and less protein and that Kieselguhr adsorbs relatively more protein and less toxin from the supernatant solution. By treating a solution of precipitate (ii) with Kieselguhr it is thus possible to purify the neurotoxin 3.9 times. This purified neurotoxin, however, still contains considerable quantities of the hemorhagin and the blood coagulating agent.

TABLE II.

Adsorbent.	Amount of adsorbent, mg.	CONTENT OF SUPERNATANT SOLUTION IN PERCENTAGE.	
		Toxicity.	Protein.
Alumina A ..	96	16.0	20.4
Alumina C ..	96	8.0	23.8
Kaolin ..	50	40.0	23.1
Fuller's earth ..	50	65.0	19.3
Kieselguhr ..	50	62.0	15.9

Elution of the toxin from the surface of aluminium hydroxide C.

In the previous section it has been mentioned that aluminium hydroxide C adsorbs relatively more of the neurotoxin and less of protein. An attempt was, therefore, made to elute the toxins from the surface of this adsorbent, after it has been treated with a solution of precipitate (ii). Fifteen c.c. of a solution containing 50,000 M. L. D. of precipitate (ii) were shaken with 10 c.c. of a suspension containing 9.6 mg. per c.c. of aluminium hydroxide C for 30 minutes. The precipitate separated from the supernatant solution by centrifuging and treated with 20 c.c. of solution containing different salts of phosphoric acid and glycerine in varying proportions. The results are recorded in Table III. It will be noticed that by using a solution of 1 per cent disodium hydrogen phosphate and 5 per cent glycerine, 40 per cent of neurotoxin associated with only 5.1 per cent of protein can be eluted. The purification effected therefore is about 7.8 times.

TABLE III.

Eluting agent.	PERCENTAGE.	
	Toxicity.	Protein.
1. 1 per cent Na_2HPO_4 + 0.5 per cent glycerine ..	10.0	..
2. 1 " " Na_2HPO_4 + 1 " " " ..	29.0	4.3
3. 1 " " Na_2HPO_4 + 5 " " " ..	40.0	5.1
4. 1 " " Na_2HPO_4 + 10 " " " ..	52.0	6.4
5. 1 " " $(\text{NH}_4)_2\text{HPO}_4$ + 1 " " glycerine + 0.01 " " NH_4OH ..	58.0	7.5

SEPARATION OF THE NEUROTOXIN FROM THE CRUDE VENOM OF
Bungarus fasciatus.

(a) *Removal of proteins by fractional precipitation.*—To 90 mg. of dry *Bungarus fasciatus* venom dissolved in 10 c.c. of water, was added sufficient quantity of a 40 per cent sodium sulphate solution so as to bring its concentration to 24 per cent in the final mixture. It was then kept in a thermostat at 37°C. for 30 minutes and then centrifuged. The precipitate was redissolved in 10 c.c. of water and reprecipitated at 24 per cent sodium sulphate concentration. The supernatant solution from this precipitation was mixed with that obtained from the previous one. The precipitate was found to contain 75.3 per cent of the protein and 26 per cent of the toxicity of the amount of venom initially taken. To the supernatant solution more of 40 per cent sodium sulphate solution was added until its concentration rose to 28 per cent. The mixture was kept in a thermostat at 37°C. for 30 minutes and then filtered. The precipitate contained 11.1 per cent of the protein and 30 per cent of the toxicity of the venom taken.

(b) *Removal of proteins by adsorption on and elution from the surface of adsorbents* :—

(i) *Tungstic acid as adsorbent.*—To the supernatant solution obtained after precipitation at 28 per cent sodium sulphate concentration, were added 1 c.c. of 2.3 N H_2SO_4 and 1 c.c. of 10 per cent tungstic acid solution. The precipitate obtained was washed once with a solution containing 1 c.c. of 2.3 N H_2SO_4 and 1 c.c. of 10 per cent solution of tungstic acid in 30 c.c. of water. The precipitate was then suspended in 10 c.c. of water and to it was added caustic soda solution until the pH rose to about 9.0. A few drops of a concentrated solution of barium chloride were then added and the mixture centrifuged. The supernatant solution was withdrawn and a few drops of 40 per cent sodium sulphate solution were added to it to precipitate the Ba-ions. It was again centrifuged and the toxicity and protein content of the supernatant liquid were determined. It was found to contain 6.8 per cent of the protein and 36 per cent of the toxicity of the venom initially used. These experiments were repeated several times and the results were found to be quite reproducible. The neurotoxin was thus purified to the extent of about 5.3 times and it was found to be free from hæmolyisin. The recovery was, however, only 36 per cent.

(ii) *Ferric hydroxide as adsorbent.*—Since the recovery of the neurotoxin was low in the experiment described above, several other methods were tried and one which proved somewhat satisfactory is recorded below. It consisted in removing the inactive proteins by repeated treatment with ferric hydroxide. The ferric hydroxide was prepared by gradually adding 1,000 c.c. of N/10 FeCl_3 , 3 H_2O solution to 1,500 c.c. of N/10 NaOH with frequent stirring. The precipitate was washed with water until free from Cl-ions and then again mixed with 1,000 c.c. of N/10 NaOH and the washing continued until the pH of the supernatant liquid was 7.2. The precipitate was then suspended in such an amount of water that 10 c.c. of the suspension contained 66 mg. of Fe_2O_3 . Ten c.c. of this ferric hydroxide suspension were added to each of a series of flasks containing 90 mg. of *Bungarus fasciatus* venom dissolved in 10 c.c. of sodium chloride solution of varying concentrations. The pH of these solutions was adjusted at 7.2 as the adsorption of the proteins was found to be the maximum between pH 7.0 and pH 8.0. The mixture was shaken for one hour and then centrifuged. The toxicity and the protein

content of the supernatant liquid were determined. The results are recorded in Table IV. It will be noticed that the best results are obtained when the concentration of sodium chloride in the mixture was about 0.75 per cent.

TABLE IV.

M. L. D. for pigeons (300 g.) = 1.8 mg.

Experiment number.	Concentration of NaCl in per cent.	CONTENT OF SUPERNATANT SOLUTION IN PERCENTAGE.	
		Protein.	Toxicity.
1	0.0	49.8	96
2	0.5	24.7	90
3	0.75	16.8	90
4	1.0	16.3	84

The supernatant solution obtained after the treatment of the venom solution with ferric hydroxide suspended at 0.75 per cent NaCl solution, was again shaken, after mixing with different volumes of the ferric hydroxide suspension. The mixtures were centrifuged and the toxicity and protein content of the supernatant were determined in each case. The results are recorded in Table V. It will be noticed that the highest purification, about 9 times, is reached when the supernatant liquid is shaken with 10 c.c. of the ferric hydroxide suspension. The recovery in this case was about 75 per cent, but the neurotoxin contained a little hæmolysin.

TABLE V.

Experiment number.	Volume in c.c. of ferric hydroxide suspension.	CONTENT OF SUPERNATANT LIQUID IN PERCENTAGE.	
		Protein.	Toxicity.
1	2	13.5	84
2	5	10.8	80
3	10	8.4	75
4	15	7.5	65

CONCLUSION.

1. A very concentrated preparation of the active principles of Russell's viper venom has been obtained by fractional precipitation with sodium sulphate followed

by adsorption on and elution from the surface of aluminium hydroxide C. For the same nitrogen content the activity of the neurotoxin in this sample was about 7·8 times greater than that in the crude venom.

2. A very active sample of the neurotoxin of *B. fasciatus* venom free from hæmolysin has been prepared by fractional precipitation with sodium sulphate, followed by adsorption on and elution from the surface of tungstic acid. For the same nitrogen content the activity of the neurotoxin in this sample was about 5·3 times greater than that in the crude venom.

3. It has also been found that by repeated treatment of a solution of *B. fasciatus* venom with ferric hydroxide in presence of 0·75 per cent NaCl and at pH 7·2, a sample is obtained in which the activity of the neurotoxin for the same nitrogen content is about 9 times greater than that in the crude venom. The neurotoxin sample, however, contains some hæmolysin.

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TETRACHLORETHYLENE IN THE TREATMENT OF HOOKWORM DISEASE, WITH SPECIAL REFERENCE TO TOXICITY.

BY

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THE problem of the treatment of anchylostomiasis is not that of the individual case, but of its mass treatment. The usefulness of a drug from this point of view depends not only on its efficacy, but also on its suitability for general use and freedom from toxicity under conditions of mass treatment. β -naphthol, thymol, chenopodium, carbon tetrachloride, and hexylresorcinol, though useful for hospital use, have proved not quite satisfactory for mass treatment. The search for a safe, efficient, easily administrable, and inexpensive drug continues.

A drug that has recently come into general use is tetrachlorethylene. It is a chlorine hydrocarbon compound, having the formula C_2Cl_4 , specific gravity 1.6, chlorine content 85.5 per cent. It is a heavy liquid, and is fairly stable in all climatic conditions. It is very insoluble in water, 1 : 10,000 (Chopra, 1936).

REVIEW OF LITERATURE.

Hall and Shillinger (1925) introduced the use of tetrachlorethylene as an anthelmintic. They found it an effective anthelmintic in dogs. Lambert (1933) treated 13 human cases with the new drug and found it has the same selective action on the female necator as does carbon tetrachloride. Schapiro and Stoll (1927) and Soper (1926) reported further successful trials with the new drug. Lamson, Robins and Ward (1929) studied the pharmacology and toxicology of tetrachlorethylene, based on animal experiments. They found that it behaved entirely differently from carbon tetrachloride. While the latter produced liver damage and even fatal results in unhealthy individuals, tetrachlorethylene was absorbed very little, if at all, from the intestinal tracts of dogs in the absence

of fat. Even in cases where, as a result of massive dosage or abnormal absorption, toxic results supervened, they were those of an overdose of a hypnotic and not those of liver damage. Very large doses failed to produce injury to the liver and kidneys. They concluded that the chances of its toxicity in human beings were very slight.

Maplestone and Chopra (1935) studied its toxicity in cats. They administered the drug in doses varying from 0.5 c.c. up to 4.5 c.c. per kilo of body-weight. When the 1.0-c.c. dose was reached and exceeded, they found that the animals exhibited giddiness, restlessness, drowsiness, and a certain amount of unsteadiness in the hind legs, the severity of the symptoms depending on the dose. However, these symptoms usually disappeared within three days. A dose of 5.0 c.c. per kilo of body-weight resulted in death in every case, usually within 24 hours. They also found that the severity of the symptoms produced was not materially affected by giving the drug in milk or water, nor was increase of toxicity observed by administering it in alcohol. They concluded that the toxic effects of the drug on human beings will be negligible, a 3-c.c. to 4-c.c. dose in an average Indian weighing 60 to 70 kilos would at most represent only 0.15 c.c. per kilo of body-weight.

Kendrick (1929), who had carried out 1,500 treatments with tetrachlorethylene in the Madras jails, however, reported one case, where severe symptoms of toxicity were observed after a dose of 3 c.c. In this case the patient became unconscious after receiving the drug, and remained in that state for about three hours. After this he rapidly recovered and in two hours was apparently normal again. The symptoms in this case were those of an overdose of a hypnotic, as Lamson and others had found in animals. He also found that tetrachlorethylene was just as efficient as any drug or combination of drugs against hookworm. A number of other workers have also reported favourably on the anthelmintic efficacy and freedom from toxicity of tetrachlorethylene in human cases. Sharp (1930) reported the experience of several workers in addition to that of his own. Maplestone and Mukerjee (1929) first used the drug in 1929 in gelatine capsules, when they did not find it superior to carbon tetrachloride, but in subsequent trials, when they gave the drug in magnesium sulphate solution (Maplestone and Mukerjee, 1933, 1937); they found that it was safer than carbon tetrachloride, on account of its non-toxicity to the liver and kidneys in therapeutic doses, and also owing to the fact that alcohol did not increase its toxicity, a very valuable advantage for mass treatment of large labour forces. They also found that in full adult doses of 4 c.c., 67.7 per cent of patients treated were practically cured after one treatment and 85.5 per cent after two treatments. Forty-six thousand treatments with tetrachlorethylene have been administered in the South Pacific Islands under the supervision of Lambert (*loc. cit.*) in four years. No deaths have resulted and it has caused fewer toxic symptoms than any of the anthelmintic drugs; and Faust (1937) reported that 100,000 treatments have been given without a death.

The review of the available literature indicates that tetrachlorethylene is an effective anthelmintic against the hookworm and that there is a wide margin of safety between the therapeutic and the toxic dose, at least as far as healthy animals and human beings are concerned. Further information is necessary as to how patients suffering from concomitant other diseases, or those with secondary organic changes in the heart, liver, kidneys, etc., react to the drug. In the mass treatment

of the disease it is not always possible to exclude this type of patient; experience with carbon tetrachloride has shown that an anthelmintic drug, which has been quite innocuous in the case of healthy subjects, has produced severely toxic and even fatal results in those who were the subjects of obvious or latent disease.

This paper gives the results of an investigation in which an attempt has been made to find the most effective dose that may be given with safety to patients admitted primarily for the treatment of conditions other than anchylostomiasis. It was carried out in two sets of patients, one a group of children under ten years of age at the Lady Ridgeway Hospital for Children, Colombo, and the other adults and older children at the General Hospital, Colombo. Both were in the wards at these two institutions under the care of the senior author.

Table I gives the age and sex distributions of the patients, and Table II the diseases that were encountered in addition to the helminthiasis. The drug was given shaken up in *mistura alba*, taking care to see that it was broken up into fine droplets. The dose of *mist. alba* was three ounces for adults and a proportionately smaller dose for children. No special preparation was made, except omitting the morning meal, the drug being given on an empty stomach, followed by a light meal at noon. Treatment was given at a stage in the illness, when it was considered safe to give the drug.

TABLE I.
Age and sex distribution.

Age (in years).	Males.	Females.	TOTALS.
Under 1 ..	0	0	0
1 to 2 ..	0	3	3
2 to 4 ..	8	3	11
4 to 6 ..	16	10	26
6 to 8 ..	13	10	23
8 to 10 ..	3	6	9
10 to 15 ..	4	1	5
15 to 20 ..	1	0	1
20 to 30 ..	12	5	17
30 to 40 ..	5	1	6
40 to 50 ..	2	2	4
50 to 60 ..	3	2	5
60 to 70 ..	1	0	1
TOTALS ..	68	43	111

TABLE II.

Diseases encountered.

Serial number.	Disease.	Number of cases.	Serial number.	Disease.	Number of cases.
1	Anæmia	9	21	Malaria	7
2	Advanced anæmia	7	22	Nephritis	2
3	Acute rheumatic fever	3	23	Epilepsy	2
4	Pulmonary tuberculosis	1	24	Influenza	3
5	Tuberculous arthritis	1	25	Chronic ulcer	1
6	Paralysis (infantile)	3	26	Paronychia	1
7	Malnutrition	2	27	Progressive muscular atrophy	2
8	Nutritional œdema	2	28	Precordial pain	1
9	Vomiting and diarrhœa	4	29	Mitral valvular disease	3
10	Rickets	1	30	Congestive heart failure	2
11	<i>Otitis media</i>	2	31	Purpura hæmorrhagica	2
12	Worms	3	32	Neuritis	1
13	Cellulitis	2	33	Tabes dorsalis	1
14	Broncho-pneumonia	4	34	Pleural effusion	1
15	Lobar-pneumonia	1	35	Jaundice	3
16	Typhoid	2	36	Cervical rib	1
17	Continued fever	2	37	Pyloric obstruction	1
18	Scabies	1	38	Plummer-Vinson syndrome	1
19	Sciatica	2	39	Hypertensive heart disease	1
20	Dog bite (Pasteur treatment)	22			

THE ANTHELMINTIC VALUE OF TETRACHLORETHYLENE.

A. *Hookworm.*

In order to assess with accuracy the anthelmintic value of any drug it is necessary to have the following data:—

- (1) The number of worms infesting the patient.
- (2) The number of worms removed by treatment.
- (3) The number of worms remaining after treatment.

Only the second of these can be ascertained by direct examination, the remaining two having to be obtained by indirect methods. One method in general use is to estimate the number of worms from the egg-count per gramme of faeces. Authorities differ widely in their estimate of the worm/eggs ratio. Figures as different as 20, 44, and 100 have been suggested as the number of eggs that correspond to an adult hookworm. Hence considerable error is possible and only approximately correct results can be obtained.

Method of investigation adopted.—The general plan adopted was first to examine stools for ova; if ova were present an ova-count was done. For three days after treatment all the stools passed were collected, and each day's stools were examined for worms which were counted. Ten days after treatment the stools were again examined for ova and an ova-count done. It was not possible to do all the examinations in every case. Hence some of the cases where the relevant data were not available had to be rejected from certain of the calculations, although included in others. This would explain the variations in the figures under the same headings in the different tables given. A 'complete cure' for the purposes of this investigation is one in which there were no ova in the stools ten days after treatment. A 'partial cure' is one in which there has been reduction of worms or eggs as a result of treatment. It has been attempted to estimate the extent of this reduction by egg-counts before and ten days after treatment, and correlating the information so derived with the results of worm-counts after treatment.

Tables III and IV give the percentage of complete cures and the average number of worms passed for the various doses for adults and children respectively:—

TABLE III.

Adults.

Dose of C_2Cl_4 in c.c.	Number examined for ova 10 days after treatment.	Complete cures.	Percentage of complete cures.	Total number of worms passed.	Number examined for worm- count for 3 days after treatment.	Average number of worms.
3 and under	9	4	44.44	396	9	44
4	20	9	45.00	1,944	20	97
6	6	4	66.60	506	6	84
8	6	6	100.00	1,198	6	199
TOTALS ..	41	23	56.00	4,044	41	101

TABLE IV.

Children.

Dose of C_2Cl_4 in minims as a multiple of the age.	Average dose in minims.	Number examined after 10 days for ova in stools.	Complete cures.	Percentage of complete cures.	Total number of worms passed.	Number where worm-count was done.	Average number of worms.
Age \times 2 ..	6.0	2	0	..	31	4	8
Age \times 3 ..	24.0	5	1	20.0	149	8	18
Age \times 4 ..	26.5	22	12	54.5	1,322	33	40
Age \times 5 ..	29.0	14	4	} 33.3	1,699	28	60
Age \times 6 ..	30.0	1	1				
TOTALS	44	18	40.9	3,201	73	43.7

Table V gives the results of 33 cases where ova-counts were done before treatment and the worms expelled after treatment were counted. The worm/ova ratio used in the calculation was 1 : 40 after Rogers and Megaw (1935).

TABLE V.

Dose of C_2Cl_4 in c.c.	Number examined.	Total number of worms present as calculated.	Average present.	Total number of worms expelled.	Average expelled.	Percentage reduction.
3	6	2,062	387	276	46	13.1
4	15	2,940	196	1,421	95	48.3
6	6	825	137	506	84	61.5
*8	6	1,050	175	1,198	199	100.0
TOTALS ..	33	6,877	..	3,401	..	49.4

* The correct worm/ova ratio appears to be 1 : 35.

It would appear from the above that the anthelmintic efficiency of C_2Cl_4 varies directly with the size of the dose. The percentage of 'complete cures' rises steadily from 44.5 per cent in the case of 3-c.c. and under doses, to 100 per cent in the case of 8-c.c. doses. In the cases of children there is an anomaly, a

dose of four times the age producing a higher percentage of 'complete cures' than one of five times the age. This is readily explained if the larger dose had not been so well tolerated as the smaller dose. But no such result was observed, both doses being equally well tolerated. An examination of the figures for the number of worms passed shows that this anomaly is only apparent and not real. The number of 'complete cures' in any given series is apt to vary not only with the efficiency of the drug, but also with the relative proportion of severe and moderate infections; a greater proportion of the latter producing a higher percentage of 'complete cures' and vice versa. This fallacy can be eliminated by studying the number of worms expelled, a more efficient dose expelling more worms in the severe infections even though the percentage of complete expulsions be less. An examination of the tables shows that there is a steady increase in the average number of worms passed as the dose increases; a dose of five times the age in the case of children expelling 50 per cent more worms than one of four times the age. In the 18 cases among children where a complete cure was effected, the total number of worms passed was 514, with an average of 27; in the 26 cases of 'partial cure' a total of 2,687 worms was passed, with an average of 103. The cure was partial in the latter group, because the infection was heavier. In the case of the adult series, the increase in the average number of worms expelled follows closely the increase in the dose and the increase in the percentage of 'complete cures', except in the case of the 6-c.c. dose, where the average number of worms expelled is a little less than that of the 4-c.c. dose. Even here the percentage reduction is greater for a 6-c.c. dose than for a 4-c.c. dose, the percentage reduction rising proportionately with the increase of dose. Maplestone and Mukerjee (1932) have criticized the attempts at estimating the percentage reduction of worms from egg-counts after treatment. The method is subject to considerable error, but, taken along with other methods, useful results may be derived from it.

Table VI gives the results of egg-counts before and ten days after treatment:—

TABLE VI.

BEFORE TREATMENT.				AFTER TREATMENT.			
Dose of C_2Cl_2 in c.c.	Number examined.	Total egg-count.	Average per patient.	Total egg-count.	Average remaining.	Average number of eggs removed.	Percentage egg reduction.
3 and under	Sufficient	number not done.			
4	15	117,600	7,800	42,000	2,800	5,040	64.2
6	6	33,000	5,500	14,400	2,400	3,100	56.4
8	6	42,000	7,000	0	0	7,000	100.0

In this table the results are not uniform, but even here the better results are obtained with higher doses. When the 8-c.c. dose is reached the worms are completely expelled, the number passed corresponding to the calculated worm-count if the ratio 1 : 35 be used, no ova being found in the stools ten days subsequent to treatment. The highest number of worms passed on this dose in one case was 510.

B. *Round worms.*

Table VII gives the results in children in terms of positive ova cases and the number of worms passed :—

TABLE VII.

Dose of C_2Cl_4 in minims as multiple of age.	Number of positive ova cases.	Total number of worms expelled.	Average number of worms passed (approximately).
Age \times 2 ..	4	7	2
Age \times 3 ..	4	36	9
Age \times 4 ..	25	252	10
Age \times 5 ..	20	202	10
Age \times 6 ..	1	40	40
TOTAL ..	54	537	10

Table VIII gives the same data for adults :—

TABLE VIII.

Dose of C_2Cl_4 in c.c.	Number of positive ova cases.	Total number of worms expelled.	Average.
3 and under	9	0	..
4	11	3	..
6	3	0	..
8	2	0	..

Table IX gives the results, in the adult series, of egg-counts before and ten days after treatment, and the number of worms expelled :—

TABLE IX.

Dose of C_2Cl_4 in c.c.	Number examined.	Total round worms ova before treatment.	Ova-count 10 days after treatment.	Percentage reduction.	Worms expelled.
3 and under	4	151,500	103,200	31.9	0
4	9	69,900	37,500	46.3	3
6	2	46,500	0	100.0	0
8	2	20,700	12,600	39.1	0

The above tables show that tetrachlorethylene has a slight action on round worms, particularly in children. Its effect, however, seems to be more to inhibit the laying of eggs rather than to expel the worms. Even 8-c.c. doses in adults had very little vermifuge effect.

Conclusions.—The following conclusions may be drawn:—

- (1) Tetrachlorethylene is an effective drug against the hookworm.
- (2) The efficiency of the drug increases with increase in the size of the dose.
- (3) The most effective dose was 8 c.c. for adults, where a 100 per cent expulsion of worms can be effected by a single treatment in patients harbouring anything up to 510 worms.
- (4) Tetrachlorethylene has little action on round worms.

TOXICITY OF TETRACHLORETHYLENE.

The cardiovascular, respiratory, renal, hepatic, and nervous systems were examined before and after treatment for any evidence of toxicity. The pulse, respiration, blood-pressure, disturbances of rhythm and rate, subjective and objective nervous changes, abnormalities in the urine, etc., provide evidence of toxicity in these organs and their significance can be assessed readily. In the case of the liver, recognition of injury is more difficult. The liver has a large reserve power and by the time that clinical evidence of liver damage appears, extensive and perhaps irreparable damage to the liver has been done. Hence more sensitive methods of recognizing toxicity have to be employed if early signs of toxicity are to be recognized. Unfortunately, there is no single test for liver function, because the liver has not one function but a number of them. It is intimately connected with the metabolism of carbohydrates, proteins, and fats. Its detoxicating functions are of the utmost importance for the maintenance of life. It excretes bile pigments, synthesizes bile salts, and plays an important part in the coagulation of blood. Any one of these functions may be affected alone, while the others are unchanged. Experience has shown, however, that if all these functions, or a number of them, are investigated at the same time, it is possible to detect signs of liver damage long before any clinical manifestations occur. Although there are several tests for liver function the following were the tests found practicable to use in this investigation:—

I. Detoxicating function—bromsulphalein test.

When a dye such as bromsulphalein is injected intravenously it is removed by the liver and excreted in the bile. If the liver is damaged the dye will remain longer than normal in the circulation, the degree of retention after a given interval indicating the degree of liver damage. This is a safe and practical test for liver function. In obstructive jaundice high degrees of retention are found without indicating equivalent liver damage. If this fallacy is guarded against, the test gives reliable results.

II. Biliary functions,

(a) *Icterus index.*—The colour of the blood serum is compared with that of a solution of potassium bichromate, a 1:10,000 solution being taken as unit. The

figure obtained is called the icterus index, and forms a quantitative estimation of bilirubin in the blood. Hæmolysis, lipœmia, and carotinœmia have to be excluded.

(b) Van den Bergh reaction helps to distinguish three types of jaundice, pure obstructive jaundice (immediate, direct); hæmolytic jaundice (indirect reaction, and delayed direct); and jaundice associated with damage to liver cells (biphasic reaction). This reaction can be estimated quantitatively, and the degree of disturbance can be followed from time to time.

(c) *Bile pigment and bile salts in urine.*—Normally no bile pigment or bile salts appear in urine. If these appear in urine after the administration of tetrachlorethylene, while they were absent before, it is justifiable to consider it as evidence of hepatic injury.

(d) *Urobilin in urine.*—Normally urobilin is present in urine in very small quantities, but not in sufficient quantity to give a reaction with Ehrlich's reagent. The bilirubin that is excreted into the intestines is converted by bacterial action into colourless urobilin. These substances are in part excreted and in part absorbed. The absorbed portion is carried by the portal vein into the liver, where it is again converted into bilirubin and excreted. If the liver cells are diseased they may be unable to dispose of all the urobilin which is offered to them, in which case it is carried to the kidney and excreted in the urine. Speaking generally, the appearance of urobilin in the urine is a more delicate indication of liver damage than the presence of bilirubin (Boyd, 1935).

Two fallacies have to be kept in mind:—

1. Urobilin may be absent in the urine even in extensive liver damage if there is complete obstructive jaundice.
2. Increased bile formation, as in hæmolysis, will increase the quantity of urobilin in the urine although the liver is quite healthy.

These two conditions may be eliminated by the other tests already mentioned, and by estimating the hæmoglobin percentage. As urobilin in the urine can be estimated quantitatively, any increase in the urobilin content of the urine, in the absence of increased hæmolysis, may be taken as a fairly delicate index of the disturbance of liver cells.

These tests were carried out in the manner described by Kolmer and Boerner (1931).

III. *Glycogenic functions—lævulose-tolerance test.*

When lævulose is absorbed from the alimentary canal and reaches the portal circulation, it is almost entirely taken up by the liver, and a very little reaches the systematic circulation. If the liver is diseased, it is allowed to pass into the systematic circulation, and the blood-sugar rises. The test was done in the same way as the ordinary glucose-tolerance test, but instead of the glucose, 50 g. of lævulose was used. The degree of rise of blood-sugar above the fasting level gives the degree of renal dysfunction.

Forty patients were treated, some more than once; and most of the above tests were done before and after treatment in forty-three instances. The results are given in tabular form:—

TABLE X.

Showing the results of hepatic efficiency tests before and after treatment.

Number.	Name, age, and sex.	Diagnosis.	Dose of C_2Cl_4 .	BLOOD.						URINE.							
				ICTERUS INDEX.		BROMSULPHALEIN.		VAN DEN BERGH.		ALBUMIN.	BILE FIG-MENT.		BILE SALTS.	UROBILIN.			
				Before.	After.	Before.	After.	Before.	After.		Before.	After.		Before.	After.	Before.	After.
1	S. Pedrick, 25, M.	Anchy.; congestive heart failure.	4 c.c.	3	3	Nil	Nil	D - I -	D - I -	Nil	Nil	Nil	Nil	Nil	Nil	+ 1/20 diln.	+ 1/10 diln.
2	M. Appahamy, 35, M.	Purpura hæmorrhagica.	4 c.c.	10	7	10 per cent.	..	DD + 0.2 mg.	DD + 0.2 mg.	Nil	Nil	Nil	Nil	Nil	Nil	1/80	..
3	M. Rosaline Nona, 10, F.	Vomiting of round worms.	40 m.	3	5	2.5 per cent.	Nil	D - I -	D - I -	Nil	Nil	Nil	Nil	Nil	Nil	Traces	Nil.
			*40 m.	5	5	Nil	Nil	DD -	DD + 0.2 mg.	Nil	Nil	Nil	Nil	Nil	Nil	Nil	1/20
4	K. A. Winifred, 60, F.	Epileptiform fits	40 m.	2	2	2.5 per cent.	2.5 per cent.	D - I -	D - I -	Nil	Nil	Nil	Nil	Nil	Nil	1.3	1/20

* Second treatment, 12 days later.

Key to Table :-

D = Immediate direct.

DD = Delayed direct.

I = indirect.

+ = Positive.

- = Negative.

TABLE X—*contd.*

Number.	Name, age, and sex.	Diagnosis.	Dose of C_2Cl_4 .	BLOOD.				URINE.							
				BROMSULPHALEIN.		VAN DEN BERGH.		ALBUMIN.	BILE PIGMENT.		BILE SALTS.		UROBILIN.		
				Before.	After.	Before.	After.		Before.	After.	Before.	After.			
18	Lazarus Fernando, 52, M.	Anæmia ; jaundice.	(1) 4 c.c. (2) 4 c.c. (3) 4 c.c.	20	5	15 per cent.	2.5 per cent.	DD + 0.8 mg.	DD + 0.2 mg.	Before.	After.	Before.	After.	Before.	After.
				5	3	2.5 per cent.	Nil	DD + 0.2 mg.	D — I —	Nil	Nil	Nil	Nil	1/5	Nil
				3	3	Nil	Nil	D — I —	D — I —	Nil	Nil	Nil	Nil	1/40	1/5
19	Gunasekera, 31, M.	Advanced anæmia (Hb = 10 per cent).	(1) 4 c.c. (2) 4 c.c.	3	3	Nil	Nil	D — I —	D — I —	Nil	Nil	Nil	Nil	1/20	1/20
20	E. P. Shaw, 40, M.	Anæmia	xl m.	3	3	Nil	Nil	D — I —	D — I —	Nil	Nil	Nil	Nil	1/30	Nil.
21	Piyoris, 15, M.	Rheumatic fever	xl m.	2	3	2.5 per cent.	7.5 per cent.	D — I —	D — I + 0.2 mg.	Nil	Nil	Nil	Nil	1/10	1/10
22	N. C. Perera, 50, F.	Progressive muscular atrophy.	6 c.c.	3	3	Nil	Nil	D — I —	D — I —	Trace	Trace	Nil	Nil	Nil	Nil
23	Josephine Nona, 22, F.	Malaria	6 c.c.	3	3	Nil	Nil	D — I —	D — I —	Nil	Nil	Nil	Nil	Nil	1/10

24	Martha Hamy, 23, F.	Precordial pain.	6 c.c.	3	3	Nil	Nil	D — I —	D — I + Faint pink.	Nil	Nil	Nil	Nil	Nil	1/5	1/5
25	Simon, 28, M.	Dyspnoea on exertion ; neuritis.	8 c.c.	3	3	Nil	Nil	D — I —	D — I —	Nil	Nil	Nil	Nil	Nil	1/30	1/20
26	S. Appu, 25, M.	Fever (?)	6 c.c.	1	1	Nil	Nil	D — I —	D — I —	Nil	Nil	Nil	Nil	Nil	1/10	1/20
27	Gabriel Appu, 30, M.	Jaundice.	3 c.c.	20	15	20 per cent.	10 per cent.	D + 1.4 mg.	DD + 1.4 mg.	Nil	Nil	+	Nil	Nil	1/20	1/5
28	Podinona, 40, F.	Anæmia dysphagia.	(1) 4 c.c. (2) 4 c.c.	3	3	Nil	Nil	D — I —	D — I —	Nil	Nil	Nil	Nil	Nil	Trace	Nil.
29	Abraham Silva, 30, M.	Influenza ; quartan malaria.	8 c.c.	5	5	Nil	Nil	D — I + Faint pink.	D — I + Faint pink.	Nil	Nil	Nil	Nil	Nil	1/20	1/10
30	Simon, 30, M.	Anæmia	6 c.c. 8 c.c.	3 3	3 3	Nil Nil	Nil Nil	D — I — D — I —	D — I — D — I + Faint pink.	Nil Nil	Nil Nil	Nil Nil	Nil Nil	Nil Nil	1/40 1/40	1/40 1/5
31	Chelliah, 30, M.	Sciatica ; malaria.	8 c.c.	1	1	Nil	2.5 per cent.	D — I —	D — I —	Nil	Nil	Nil	Nil	Nil	1/10	1/40

Key to Table :—

D = Immediate direct.

DD = Delayed direct.

I = Indirect.

+ = Positive.

— = Negative.

TABLE X—concl'd.

Number.	Name, age, and sex.	Diagnosis.	Dose of C_2Cl_4 .	BLOOD.				URINE.							
				ICTERUS INDEX.	BROMSULPHALEIN.		VAN DEN BERGH.	ALBUMIN.		BILE PIGMENT.		BILE SALTS.		UROBILIN.	
				Before.	After.	Before.	After.								
32	Tharlis, 27, M.	Anytroph	8 c.c.	5	3	Nil	Nil	Before.	D — I —	Before.	Nil	Before.	Nil	Before.	1/10
								After.	D — I —	After.	Nil	After.	Nil	After.	1/20
33	H. A. P e r e r a, 32, M.	Tabes dorsalis.	8 c.c.	3	3	Nil	Nil	Before.	D — I —	Before.	Nil	Before.	Nil	Before.	1/10
								After.	D — I —	After.	Nil	After.	Nil	After.	1/20
34	W e e r a s i n g h e, 23, M.	Arthritis.	8 c.c.	5	3	Nil	Nil	Before.	DD + I + Faint pink.	Before.	Nil	Before.	Nil	Before.	1/10
								After.	DD + I + Faint pink.	After.	Nil	After.	Nil	After.	1/10
35	Danoris, 42, M.	Congestive heart failure (W.R.+).	4 c.c.	7	7	15 per cent.	10 per cent.	Before.	DD + 0.2 mg.	Before.	Nil	Before.	Nil	Before.	1/20
								After.	DD + 0.2 mg.	After.	Nil	After.	Nil	After.	1/40
36	S. A. Charles, 24, M.	Cervical rib	8 c.c.	3	3	Nil	Nil	Before.	D — I —	Before.	Nil	Before.	Nil	Before.	1/10
								After.	D — I —	After.	Nil	After.	Nil	After.	1/10
37	Albert, 9, M.	Malaria (M.T.)	xl m.	1	5	Nil	Nil	Before.	D — I —	Before.	Nil	Before.	Nil	Before.	1/5
								After.	D — I + 0.2 mg.	After.	Nil	After.	Nil	After.	1/40

38	Kolu, 51, M.	Jaundice	3 c.c.	50	20	60 per cent.	25 per cent.	BD + 4.2 mg.	BD + 2.4 mg.	+	Nil	+	Nil	+	Nil	1/20	1/10
39	Girigoris Porera, 70, M.	Duodenal ulcer	6 c.c.	1	1	Nil	Nil	D - I -	D - I -	Nil	Nil	Nil	Nil	Nil	Nil	1/10	1/10
40	Charles Singho, 24, M.	Myopathy anaemia	8 c.c.	3	3	Nil	Nil	D - I -	D - I -	Nil	Nil	Nil	Nil	Nil	Nil	1/40	1/10

Key to Table :-

D = Immediate direct.

DD = Delayed direct.

BD = Biphasic.

I = Indirect.

+

= Positive.

-

= Negative.

Lactulose-tolerance test

Charles Singho, 24, M.		Percentage before treatment.	Percentage after treatment.
(1) Fasting	..	86	93
(2) After $\frac{1}{2}$ hour	..	93	118
(3) After 1 hour	..	106	125
(4) After $1\frac{1}{2}$ hours	..	100	100
(5) After 2 hours	..	93	93

Examination of the table shows the following:—

1. *Excretory functions.*—In 40 cases where the bromsulphalein test was done before and after treatment, it was found unchanged in 26, and actually improved in 10 cases, the average improvement being 7·5 per cent and the highest 35 per cent. In four cases there was an increase in the retention of the dye. The average increase was 3·1 per cent.

2. *Biliary functions.*—Icterus index was done before and after treatment in 43 cases. In three of these there was obvious jaundice (16 units and over), but in every one of these the jaundice became less after treatment, the average diminution being by 16 units. In six cases there was latent jaundice (6 to 15 units). In four of these the jaundice diminished, in one it was unchanged, and in one it increased. This was in a patient with mitral stenosis and congestive failure, in whom the original condition was sufficient to account for the increase. In 34 cases, where the figures were within normal limits (1 to 5 units), the index diminished in 4, in 24 it remained unchanged, and in five it increased by 1 or 2 units. In another case there was an increase of 4 units, but this was due to an inter-current relapse of malaria. A difference of 1 or 2 units is difficult to read and is not of much importance either way. In the two cases where there was an increase by 3 units and 4 units respectively, one was a patient with mitral stenosis and congestive heart failure, and the other had a relapse of malaria between the two examinations. In both cases the increase can be ascribed to the original conditions.

Van den Bergh test was done both qualitatively and quantitatively in 43 cases. In no case was there any evidence of liver damage shown, as demonstrated by the appearance of a biphasic reaction. In only six cases was there a quantitative increase, the highest being one of 0·2 mg. In the remaining cases there was a decrease in the figures, or they were unchanged. In the three cases of jaundice there was considerable reduction of van den Bergh units.

In 43 cases the urine was examined before and after treatment. In no case was there bile pigment in the urine after treatment, although it was positive in the urine before treatment in three cases.

3. *Urobilinuria.*—Urobilin was estimated quantitatively before and after treatment in 42 cases. In 12 of these it was increased, in the rest it was unchanged or diminished. In three of these cases there was mitral disease and congestive heart failure, and in two others a relapse of malaria. These were the cases where the increase was considerable and the clinical conditions were sufficient to account for the increase. In the remaining eight cases the increase was very slight, being only one dilution more than the previous figure.

4. *Glycogenic functions.*—A lævulose-tolerance test was done before and after treatment in the case where the highest dose per kilo of body-weight was given. Although the blood-sugar was increased by 32 mg. in the second test, compared with 20 mg. in the first test, the highest blood-sugar reading was only 125 mg., and the blood-sugar came down to the fasting level an hour later. There was thus no certain indication of hepatic damage (Rolleston and McNee, 1929).

5. *Renal functions.*—In 43 instances the urine was examined for albumin before and after treatment. In no case was there albumin after treatment, nor was there any clinical sign pointing to renal involvement.

When all the tests are considered together, in no case is there reason to think that liver function was affected appreciably. The tests show disturbances in one function or other in a few cases. The figures are, however, well within the limitations of technical error or normal variations in patients who are ill with other pathological conditions. In the nine patients where there was already evidence of liver dysfunction, the exhibition of tetrachlorethylene failed to aggravate the condition in any one of them.

Christinsen and Lynch (1933) had found that tetrachlorethylene caused considerable depression of the heart and respiration in dogs, when given in therapeutic doses. To investigate this, in 11 patients who received tetrachlorethylene in doses varying from 6 c.c. to 8 c.c., the pulse, respiration, blood-pressure, and mental and general conditions were noted at intervals for several hours after treatment. The results are given in Table XI.

In four patients there was giddiness coming on half to one hour after taking the drug; two of these felt faint, one patient became drowsy, dull and semi-conscious, and remained so for about three hours, after which period he fully recovered and was normal again in about four and a half hours of taking the drug. In the other patients the faintness and giddiness were of comparatively short duration and all the patients had recovered fully by noon. In only one case was there any appreciable effect on the blood-pressure noted; here it fell from 135/80 to 112/70 at the end of one hour after treatment. The normal blood-pressure, however, is subject to variations. No untoward effects were noted in any patient receiving 4 c.c. or less, and the only serious case of a narcotic effect was in the patient who received 8 c.c., equivalent to 0.21 c.c. per kilo of body-weight.

It may be concluded that in the adult patients:—

- (a) No appreciable toxic effect on the liver was noted in any one of them, even in those receiving double the maximum adult dose in general use.
- (b) No depressant effects of any importance on the cardiovascular and respiratory systems were noted in any one even after 8-c.c. doses.
- (c) There was no toxic action on the kidneys.
- (d) A few of them who received doses of 6 c.c. or more showed untoward effects. These were of the nature of an overdose of a narcotic drug. These effects were only temporary.

TOXICITY IN CHILDREN.

In the case of children it was found not practicable to do all the hepatic efficiency tests that were done in the adults. Reliance was placed on the results of urine examination for albumin, bile pigment, bile salts, and the quantitative estimation of urobilin. The last was correlated with hæmoglobin percentage.

TABLE XI.

Showing the results of clinical observations before and at intervals after treatment.

Number.	Name, age, sex, and weight.	Dose of C ₆ Cl ₄ and dose per kilo of body- weight.	PULSE, RESPIRATION, BLOOD-PRESSURE, AND TOXIC SYMPTOMS.				
			Before treatment.	$\frac{1}{2}$ hour after treat- ment.	1 hour after treatment.	$1\frac{1}{2}$ hours after treat- ment.	2 hours after treat- ment.
1	Martha Hamy, 23, F., 98 lb.	6.0 c.c.	P/R = 100/28	P/R = 96/24	P/R = 96/40	Feeling better	P/R = 94/36
		0.13 c.c.	B.P. = 124/80	B.P. = 118/80	B.P. = 130/86	B.P. = 130/80
					Vomited. Feeling of giddiness and faintness for half an hour.		Feeling normal.
2	N. C. Perera, 50, F., 80 lb.	6.0 c.c.	P/R = 80/22	P/R = 90/24	P/R = 84/22	Nil abnormal	P/R = 90/24
		0.16 c.c.	B.P. = 150/100	B.P. = 150/96	B.P. = 160/100	B.P. = 158/100
3	Josephine Nona, 22, F., 80 lb.	6.0 c.c.	P/R = 84/16	P/R = 74/16	P/R = 80/16	P/R = 80/18
		0.16 c.c.	B.P. = 108/60	B.P. = 102/60	B.P. = 100/58	B.P. = 106/60
4	H. A. Perera, 32, M., 104 lb.	8.0 c.c.	P/R = 88/44	P/R = 86/44	P/R = 80/40	P/R = 88/36	P/R = 86/36
		0.17 c.c.	B.P. = 104/77	B.P. = 108/76	B.P. = 108/80	B.P. = 104/76

5	Werasinghe, 23, M., 112 lb. {	8.0 c.c. 0.157 c.c.	P/R = 52/18 B.P. = 100/64	Nil abnormal	P/R = 54/18 B.P. = 100/62	P/R = 60/14 B.P. = 100/60
6	A. Silva, 30, M., 104 lb. {	8.0 c.c. 0.17 c.c.	P/R = 70/? B.P. = 100/65	P/R = 76/30 B.P. = 100/68 Feels sick and giddy. Started about 15 minutes after taking medicine. No vomiting.	P/R = 72/24 B.P. = 100/72 Feeling of sickness dis- appearing. Feeling better.	P/R = 72/24 B.P. = 94/70	P/R = 74/24 B.P. = 100/70
7	Seemon, 28, M., 78 lb. {	6.0 c.c. 0.169 c.c.	P/R = 80/30 B.P. = 104/64	P/R = 84/26 B.P. = 104/60	P/R = 80/24 B.P. = 102/60	P/R = 80/22 B.P. = 104/60
8	Girigoris Perera, 70, M., 90 lb. {	6.0 c.c. 0.147 c.c.	P/R = 66/24 B.P. = 108/74	P/R = 68/20 B.P. = 122/74	P/R = 70/16 B.P. = 120/80 Feeling giddy.	P/R = 76/20 B.P. = 116/80 Feeling giddy.	P/R = 72/22 B.P. = 108/76 Feeling of giddiness less. Feeling ex- tremely weak.
9	S. Chelliah, 30, M., 120 lb. {	8.0 c.c. 0.147 c.c.	P/R = 72/18 B.P. = 86/46	P/R = 72/18 B.P. = 82/44	P/R = 64/16 B.P. = 84/48	P/R = 64/18 B.P. = 84/50

Key to Table :-

P = Pulse.

R = Respiration.

B.P. = Blood-pressure.

TABLE XI—*concl'd.*

Number.	Name, age, sex, and weight.	Dose of C ₂ Cl ₄ and dose per kilo of body- weight.	PULSE, RESPIRATION, BLOOD-PRESSURE, AND TOXIC SYMPTOMS.			
			Before treatment.	½ hour after treat- ment.	1 hour after treatment.	1½ hours after treat- ment.
10	Samuel Appu, 58, M., 86 lb.	6.0 c.c.	P/R = 60/32	P/R = 60/30	P/R = 60/26	P/R = 64/24
		0.15 c.c.	B.P. = 135/80 Patient is feeling a bit giddy; has ringing sounds in ears.	B.P. = 124/76 Still feels a bit giddy.	B.P. = 112/70	B.P. = 108/66 Feeling of giddiness much less.
11	Charles Singho, 24, M., 84 lb.	8.0 c.c.	P/R = 70/18	P/R = 62/14	Patient unable to see. Tried to go to the bathroom, but found he could not walk.	P/R = 64/16
		0.21 c.c.	B.P. = 100/60	B.P. = 96/58 Feeling giddy and faintish.	B.P. = 98/56 Patient is drowsy and dull. Does not answer questions.	B.P. = 98/58 Patient very dull and drowsy; unable to respond directly to questions asked. Quite normal at 12:30 (i.e., 4½ hours after treatment).

Key to Table :—
P = Pulse.

R = Respiration.

B.P. = Blood-pressure.

Seventy patients below the age of ten years were given tetrachlorethylene along with *mist. alba*. Eighty-two treatments were given. The dose in minims was a multiple of the age.

1. Age \times 2	4 treatments.
2. Age \times 3	8 "
3. Age \times 4	36 "
4. Age \times 5	34 "

- (a) In no case was there any clinical evidence of toxicity; the drug was well tolerated and there was no nausea, vomiting, signs of intoxication, or collapse.
- (b) In 67 cases the urine was examined for albumin before and after treatment; in 65 cases no albumin was noted after treatment. In two there was a trace of albumin in the urine after treatment.
- (c) In 65 cases the urine was examined for bile pigment and bile salts before and after treatment, and not in one case was there any increase of these after treatment.
- (d) In 65 patients urobilin was estimated quantitatively before and after treatment. In the great majority of the cases the urobilin content was unchanged or diminished. Thus, in 24 cases the urobilin content decreased, in 25 it remained unaffected, and in 16 it was increased. In seven of the last there was a diminution of the hæmoglobin percentage to explain the increased urobilinuria.

The figures for the remaining nine are as follows:—

Case number:—	1	2	3	4	5	6	7	8	9
Before treatment.	1/30 dilution	<i>Nil</i>	<i>Nil</i>	<i>Nil</i>	<i>Nil</i>	<i>Nil</i>	<i>Nil</i>	1/20 dilution	1/20 dilution
After treatment.	1/40 dilution	1/30 dilution	1/10 dilution	1/20 dilution	1/20 dilution	1/10 dilution	1/10 dilution	1/50 dilution	1/50 dilution

It would be seen that in the majority of the cases the increases are very slight. Of the nine cases of increase the distribution for dosage was as follows:—

Number of times the age.	Number treated.	Number of cases with urobilin disturbance.
		Per cent
4	36	5 = 14.0
5	34	2 = 5.9
3	8	2 = 25.0

The results in children may be summarized thus:—

1. In no case was there any clinical evidence of toxicity, although doses up to five times the age in minims had been given.
2. There was hardly any evidence of renal damage.
3. Biliary functions showed no abnormality in any case.
4. In 13·8 per cent of cases there was a slight disturbance of urobilin function. In 37 per cent of cases there was distinct improvement after treatment, and in the others it was unchanged.

SUMMARY AND GENERAL CONCLUSIONS.

1. The available literature on tetrachlorethylene has been reviewed.
2. The anthelmintic activity of tetrachlorethylene has been studied in 111 patients, ranging in age from 2 years to 70 years.
3. The drug was found to be effective against hookworm, a dose of 8 c.c. being the most effective dose.
4. It had a slight action against round worms.
5. The toxicity of tetrachlorethylene has been studied with special reference to liver damage. No appreciable toxic action has been found even after 8-c.c. doses on the cardiovascular, respiratory, hepatic, and renal organs.
6. In a few cases, doses of 6 c.c. and more have produced symptoms of giddiness, faintness, and weakness, and in one case that of narcosis. The effects were only temporary.
7. Doses of 4 c.c. to 5 c.c. may be safely given to adult patients, and two such treatments at intervals of ten days may be expected to cure even severe cases of anchylostomiasis. Children may be given tetrachlorethylene in doses of four to five times the age in minims.
8. Tetrachlorethylene, in view of its low toxicity and efficacy, is a very valuable drug in the mass treatment of anchylostomiasis.

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CANCER IN INDIA.

BY

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IN our papers on the subject of 'Cancer in India' published in this *Journal* in July 1935 and in January 1937 (Nath and Grewal, 1935, 1937), we set forth the results of our investigations concerning the provinces of the Punjab, Delhi, the United Provinces, Behar & Orissa, Burma, and Madras. We now furnish data on the same lines from the central belt comprising the provinces of Assam, Bengal, the Central Provinces, and Bombay including Sindh. The Indian States of Rajputana, Kathiawar, Central India, Hyderabad, and Mysore are also included in this survey. In regard to these States and the smaller provinces of Assam, the Central Provinces, and Sindh, the autopsy and pathological histology data were too meagre to offer a basis of comment. Tables II, VI-c, and VI-d will, however, furnish such statistical information as was available from these areas.

In Bengal autopsy records of 3,600 cases in all were studied. This series furnished 166 cases of malignant disease, giving an incidence of 1 in 21·7 (Table I-b). In the Bombay Presidency records of 10,820 autopsies were studied. Three hundred and seventy-six cases of malignant disease were found in this series, giving an incidence of 1 in 28·7 (Table I-a). The total figure for autopsy records studied in this survey is 14,774, of which 548 were cases of malignant disease. Incidence in this case would work out to 1 in 26·9 (Table I). Table II will show regional distribution of malignant disease in the autopsy data taken as a whole and according to different institutions visited. In this series the sites of incidence of cancer in order of frequency are as follows :—

Gastro-intestinal tract 54·3 per cent, buccal 6·7 per cent, female genitals 6·0 per cent, penis 2·6 per cent, breast 1·4 per cent, and skin 0·7 per cent. On

comparative study of data collected from different institutions in Bombay and Bengal (Tables III-*a* and III-*b*), noteworthy variations in the sites affected are as follows:—

Cancer of the gastro-intestinal tract, although representing the site of maximum incidence both in Bombay and Bengal, represents higher incidence in autopsy material of the latter presidency than the former (72.0 per cent).

The second place in order of frequency in autopsy material in Bombay is occupied by buccal cancer (7.8 per cent), while the same place in Bengal is occupied by the cancer of the female genital tract (4.5 per cent).

Table III will show the incidence of cancer in autopsy cases according to communities and sex. No significance can attach to the higher figure of incidence of cancer amongst Hindus, because in the area surveyed Hindus were on the whole in a majority. The regional distribution, however, carries certain peculiarities.

Buccal cancer was commoner amongst Mohammedans. That community was, however, virtually exempt from penile cancer. The autopsies on Mohammedan women were too few to be the subject of any deductions.

In the matter of age incidence of cancer (Table IV), the autopsy records furnished the basis for the same observation as we have already made in our previous papers, viz., that the age of maximum incidence of cancer is from 40 to 50 and not from 50 to 60 as is the case in Japan, most of the European countries and the United States of America; that cancer of the female genital tract begins to show high incidence in the third decade of life, reaching its maximum before the age of 45, and that buccal cancer also shows very high incidence in the third decade of life.

Morbid histology records of 9,855 cases were studied in Bombay and 6,143 in Bengal. The number of specimens diagnosed malignant in the former series was 1,848 and in the latter 1,136. It appeared that 1 out of 5.3 specimens in Bombay and 1 out of 5.4 in Bengal were malignant. The combined figure viewed as a whole (Table VI) showed the buccal cavity to be site of maximum incidence (16.3 per cent), then came breast (15.8 per cent), then the female genital tract (15.3 per cent) which was followed by penile cancer (9.4 per cent), cancer of the gastro-intestinal tract (7.1 per cent), and cancer of skin (6.9 per cent). In Bombay the regional incidence (Table VI-*b*) was in the same order: buccal (19.4 per cent), breast (16.5 per cent), female genital tract (12.6 per cent), penis (9.2 per cent), gastro-intestinal tract (7.9 per cent), and skin (2.9 per cent). In Bengal (Table VI-*a*) the order was different: female genital tract (21.8 per cent), breast (15.2 per cent), buccal (13.0 per cent), skin (11.7 per cent), penis (9.4 per cent), and gastro-intestinal tract (5.7 per cent).

Regional distribution according to sex (Tables VIII and IX) showed that buccal cancer, taking the data of entire survey into consideration, showed a higher incidence in the male (23.5 per cent) as compared with the female (8.0 per cent). In Bombay (Tables VIII-*b* and IX-*b*) the relative figures were 26.5 per cent and 11.4 per cent; in Bengal (Tables VIII-*a* and IX-*a*) 22.0 per cent and 4.3 per cent, and in the small provinces (Tables VIII-*c* and IX-*c*) 18.1 per cent and 10.2 per cent.

Buccal cancer, studied according to its incidence on communities (Table VIII-a), showed that the general distribution was about the same in all communities except that in Bengal. It showed higher incidence amongst Mohammedans (31·5 per cent) than Hindus (19·5 per cent). Other communities had as high an incidence as 28·8 per cent. Incidence of buccal cancer (Table IX-a) was higher amongst Mohammedan women than women of other communities. Cancer of gastro-intestinal tract (Tables VIII and IX) showed a heavier incidence in males (8·9 per cent) than in females (4·7 per cent). The Bombay figure (Tables VIII-b and IX-b) was 10·2 per cent male and 4·1 per cent female. In Bengal this figure (Tables VIII-a and IX-a) stood even: 5·6 per cent for both sexes. Mohammedan males showed a higher incidence of cancer of the gastro-intestinal tract. The same consideration applies to skin cancer (Tables VIII and IX), the cancer of this site being commoner in the male (9·2 per cent) than the female (4·4 per cent). In Bombay the proportion (Tables VIII-b and IX-b) was 4·7 to 0·9 and in Bengal (Tables VIII-a and IX-a) 16·0 to 6·8. In Bombay the incidence was the highest on the Mohammedan community and in Bengal amongst Hindus. As for cancer of the breast (Table IX-b), it showed its highest incidence amongst Mohammedan females both in Bombay (51·5 per cent) and in Bengal (Table IX-a); 41·2 per cent against a combined incidence for women of all communities 35·0 per cent in Bombay and 26·7 per cent in Bengal. So far as cancer of the female genital tract is concerned (Table IX), it showed a higher incidence amongst Hindu women 39·9 per cent as against 22·0 per cent amongst Mohammedan women. In Bombay (Table IX-b) this ratio is as 34 : 22·7 and in Bengal (Table IX-a) as 47·9 : 25·8. Penile cancer showed a negligible incidence amongst Mohammedans of 1·8 per cent (Table VIII) as compared with Hindus (22·2 per cent). In Bombay this proportion (Table VIII-b) was as 2·5 : 20·2 and in Bengal (Table VIII-a) as 1·8 : 24·2.

Records of 464,227 in-patients treated mostly in the hospitals attached to medical colleges and schools in the area surveyed (Table XI) revealed the incidence of 7,570 cases of malignant disease. Of this the institutions visited in the Bombay Presidency gave 2,896 cases of malignant disease out of a total of 153,260 cases treated for all diseases. In Bengal 2,401 cases were found out of 219,540; in the small provinces 570 out of 46,189 and in the States 469 out of 45,238. This gives an incidence of 1 in 52 in Bombay, 1 in 91 in Bengal, 1 in 73 in the small provinces, and 1 in 96 in the States. In the area surveyed taken as a whole the incidence worked out as 1 in 61.

Considering the clinical data of this survey collectively, the sites affected in order of frequency are as follows:—

Table XI—buccal (24·3 per cent), gastro-intestinal tract (19·8 per cent), female genital tract (14·2 per cent), breast (9·9 per cent), penis (5·7 per cent), and skin (4·6 per cent). The Bombay figures taken by themselves (Table XI-a) show the same order: buccal (23·7 per cent), gastro-intestinal tract (22·2 per cent), female genital tract (16·2 per cent), breast (8·8 per cent), penis (5·2 per cent), and skin (2·1 per cent). In Bengal (Table XI-b) the order was different: gastro-intestinal tract (26·3 per cent), female genital tract (16·2 per cent), breast (10·9 per cent), buccal (9·9 per cent), skin (7·9 per cent), and penis (6·9 per cent). In small provinces (Table XI-c) gastro-intestinal tract (22·6 per cent), breast (13·7 per cent), buccal (13·05 per cent), female genital tract (12·2 per cent), skin (10·2 per cent), and penis (5·6 per cent). In the

States (Table XI-d): buccal (47.3 per cent), female genital tract (9.4 per cent), breast (9.3 per cent), gastro-intestinal tract (6.9 per cent), penis (5.1 per cent), and skin (2.8 per cent).

Studied in its distribution between the two sexes (Tables XIII and XIV), it was found that buccal cancer was commoner in the male (32.8 per cent) than the female (12.2 per cent). Same was true of cancer of gastro-intestinal tract (male 24.0 per cent, female 13.2 per cent) and skin (male 5.1 per cent, female 3.6 per cent). This order of preponderance was borne out when figures for different areas were considered separately. In Bombay (Tables XIII-a and XIV-a) the proportion of buccal cancer in the male to that in the female was 33.5 : 11.7, gastro-intestinal tract 30.1 : 12.5, and skin 3.1 : 0.9. In Bengal (Tables XIII-b and XIV-b) gastro-intestinal tract 33.5 : 17.9, buccal 13.4 : 5.7, and skin 8 : 7.5. In small provinces (Tables XIII-c and XIV-c) gastro-intestinal tract 29.7 : 11.7, buccal 16.2 : 8.4, and skin 14 : 2.8. In the States (Tables XIII-d and XIV-d) buccal 65.5 : 21.7, gastro-intestinal tract practically the same in both sexes 6 : 6.2 and skin 3.1 : 2.1.

When the combined data are considered as regards the incidence of malignant disease in different communities (Table XIII), the incidence of penile cancer is found to be the highest in the Hindu community (12.1 per cent), least amongst the Mohammedans (1.8 per cent) and other communities occupy an intermediate position (5.4 per cent). Cancer of the female genital tract (Table XIV) behaves in the same way. Its highest incidence is on Hindu females (39.0 per cent), least on Mohammedan women (26.3 per cent) and other communities hold an intermediate position (35.4 per cent). Cancer of the gastro-intestinal tract (Table XIII) is proportionately the highest amongst males of other communities (34.7 per cent), the lowest amongst Hindu males (21.6 per cent) and the Mohammedan males occupy an intermediate position (28.9 per cent). Amongst females (Table XIV) other communities again showed the highest incidence (15.9 per cent) but Hindu females came next (13.0 per cent) and Mohammedan females showed 11.6 per cent. As for buccal cancer (Table XIII), Hindu males show 33.5 per cent, Mohammedan males 31.6 per cent and males of other communities 28.9 per cent. Mohammedan females (Table XIV), however, show 19.6 per cent, Hindu females 10.1 per cent, and females of other communities 7.4 per cent. As to cancer of the skin (Table XIII), Mohammedan males show 7.1 per cent, Hindu males 4.7 per cent, and males of other communities 3.2 per cent. For the same site (Table XIV) women of other communities show 5.9 per cent, Hindu females 3.4 per cent, and Mohammedan females 2.3 per cent. Cancer of the breast (Table XIV) is the highest amongst Mohammedan women (30.5 per cent), the lowest amongst Hindu women (22.4 per cent), and other communities come in between (26.1 per cent).

Considering the incidence along the same lines in different provinces (Table XIII-a), Hindu males in Bombay show a penile cancer rate of 12.2 per cent, males of other communities 3.5 per cent, and Mohammedans 0.6 per cent. In Bengal (Table XIII-b) comparable figures are : Hindu males 16.1 per cent, other males 12.0 per cent, and Mohammedan males 2.5 per cent; in the smaller provinces (Table XIII-c) Hindu males 15.7 per cent, other males 6.4 per cent, and Mohammedan males 3.3 per cent; and in the States (Table XIII-d) Hindu males 8.1 per cent and Mohammedan males 2.3 per cent.

Cancer of the female genital tract (Table XIV-a) showed amongst Hindu women in Bombay an incidence of 43·5 per cent, amongst women of other communities 36·2 per cent, and amongst Mohammedan women 32·7 per cent. In Bengal (Table XIV-b) the figures for the same communities in order will be 36·0, 32·1, and 32·6 per cent; for smaller provinces (Table XIV-c) 35·7, 33·3, and 23·6 per cent; and for the States (Table XIV-d) 34·9, 52·6, and 13·0 per cent.

Cancer of the gastro-intestinal tract amongst males of other communities in Bombay (Table XIII-a) is 39·7 per cent, among Mohammedan males 29·6 per cent, and amongst Hindu males 25·9 per cent. Comparable figures for the same communities in Bengal (Table XIII-b) will be 34·5, 33·8, and 33·5 per cent; in the smaller provinces (Table XIII-c) 9·7, 31·9, and 31·8 per cent; and in the States (Table XIII-d) 33·3, 14·9, and 5·2 per cent. For the same site women of other communities in Bombay (Table XIV-a) show an incidence of 13·5 per cent, Mohammedan women 14·5 per cent, and Hindu women 11·7 per cent. Comparable figures for women of the same communities in Bengal (Table XIV-b) would be 20·7, 14·8, and 17·9 per cent; in small provinces (Table XIV-c) 20·8, 3·6, and 14·3 per cent, and in the States (Table XIV-d) Mohammedan women 9·0 per cent and Hindu women 5·7 per cent. The figures for this site relating to women of other communities in the States were too small to be considered suitable for comparison.

As for buccal cancer (Table XIII-a), Mohammedan males in Bombay show 39·6 per cent, males of other communities 29·8 per cent, and Hindu males 28·8 per cent. Comparable figures for males of these communities in the same order in Bengal (Table XIII-b) will be 14·7, 18·9, and 12·6 per cent; for smaller provinces (Table XIII-c) 15·9, 41·9, and 10·2 per cent; and for the States (Table XIII-d) 55·1, 33·3, and 67·6 per cent. Mohammedan women in Bombay (Table XIV-a) show an incidence of buccal cancer of 21·3 per cent, women of other communities 11·6 per cent, and Hindu women 9·7 per cent. Figures under the same heads for Bengal (Table XIV-b) are 12·8, 0·7, and 5·7 per cent; for the smaller provinces (Table XIV-c) 9·0, 16·6, and 6·2 per cent; and for the States (Table XIV-d) 30·0 per cent for Mohammedan women and 20·5 per cent for Hindu women.

Figures for skin cancer for different provinces vary and are of not much significance for purposes of comparison. The incidence of cancer of the breast amongst Mohammedan women in Bombay (Table XIV-a) was 20·6 per cent, women of other communities 26·5 per cent, and Hindu women 21·3 per cent. Comparable figures for communities in the same order for Bengal (Table XIV-b) were 24·7, 28·6, and 19·7 per cent; for the smaller provinces (Table XIV-c) 52·7, 8·3, and 29·4 per cent; and for the States (Table XIV-d) 37, 26·3, and 27·7 per cent.

DISCUSSION.

On the whole the findings of this survey point to the same general conclusions as have already been stated by us in our previous papers. The fact that cancer of the gastro-intestinal tract is the commonest type met with at autopsy indicates that it represents in reality the site of maximum incidence in India. In the clinical material also this type occupies a very prominent position. That in some provinces it occupies the first position in order of preponderance in the clinical material and in others not so perhaps indicates that the clinical assessment of

cancer of the gastro-intestinal tract in India varies and has not yet reached the level attained in Western countries and in Japan. In the area under review in this paper the maximum incidence of this type of cancer is found in Bengal both according to autopsy and clinical records. This observation indicates the advisability of more detailed study of causal factors in Bengal.

Buccal cancer occupies a prominent position in all types of records. In the autopsy records it stood second and in the histological and clinical records it occupied the first position. In Bengal buccal cancer does not occupy a position of similar importance. The *Pan* habit is practically as common in that Presidency as over the rest of the area surveyed and an answer will have to be found to this relative infrequency.

Penile cancer is of course a disease of the uncircumcized. Incidence among Mohammedans is negligible, but why should there be a higher incidence amongst the Hindus as against other non-muslim communities is again a problem worth studying. It is a curious coincidence that cancer of the female genital tract is also commoner amongst the Hindu women than in women of other communities. It has been observed that cancer of the female genital tract is less common amongst Jewish women than other women in the West. Cancer of the penis amongst Jews is as uncommon as amongst the Mohammedans. Is any conjugal relationship possible between penile cancer and cancer of the female genital tract, should be an interesting question and the statistics presented by us may prove of some help in its consideration.

This paper completes a general survey of the available statistics on the subject of 'Cancer in India'. To our mind the most outstanding fact with regard to incidence of malignant diseases in India is that the origin of the bulk of it lies in chronic irritation. It is, therefore, preventable through education and the attainment of higher standards of personal hygiene and public health. The survey has also shown that valuable information can be collected and presented regarding diseases, on which reports on Indian vital statistics were so far silent. The subject of cancer is of international interest and it is hoped that arrangements will be taken in hand to draw permanently on sources tapped in this survey, so that information on the incidence of cancer in India, however partial, may continue to be made available to the scientific world.

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Idem (1937) *Ibid.*, **24**, p. 633.

TABLE I.
Incidence of malignant disease as shown in the autopsy records of the following presidencies and provinces.

Name of the presidency or province.	Total number of autopsies performed.	Total number diagnosed to have malignant disease.	Tera-toma.	Carcinoma.	Sarcoma.	Endothelioma.	Hodgkin's disease.	Ratio of sarcoma to carcinoma.	Ratio of malignant disease to total number of autopsies.
Bombay ..	10,820	376	1	318	41	7	9	1 : 7.7	1 : 28.7
Bengal ..	3,600	166	..	111	43	4	8	1 : 2.6	1 : 21.7
Small provinces ..	304	1	..	1	1 : 304
The States ..	50	5	..	3	2	1 : 1.5	1 : 10
TOTAL ..	14,774	548	1	433	86	11	17	1 : 5	1 : 26.9

TABLE I-a.
malignant disease as shown in the autopsy records of the following institutions in Bombay Presidency.

name of institution.	number of autopsies performed.	Total number diagnosed to have malignant disease.	Teratoma.	Carcinoma.	Sarcoma.	Endothelioma.	Hodgkin's disease.	Ratio of sarcoma to carcinoma.	Ratio of malignant disease to total number of autopsies.
Grant Medical College, Bombay.	7,823	224	..	193	20	4	7	1 : 9.6	1 : 35
Govardhandas Sunderdas Medical College, Bombay.	2,916	150	1	124	20	3	2	1 : 6.2	1 : 19.4
B. J. M. S. Civil Hospital, Ahmedabad.	31	2	..	1	1	1 : 1	1 : 15.5
Mission Hospital, Miraj	50
TOTAL ..	10,820	376	1	318	41	7	9	1 : 7.7	1 : 28.7
Grant Medical College Medico-legal post-mortems, Bombay.	4,706	7	..	7	1 : 672

TABLE I-b.
Incidence of malignant disease as shown in the autopsy records of the following institutions in Bengal Presidency.

Name of institution.	Total number of autopsies performed.	Total number diagnosed to have malignant disease.	Teratoma.	Carcinoma.	Sarcoma.	Endothelioma.	Hodgkin's disease.	Ratio of sarcoma to carcinoma.	Ratio of malignant disease to total number of autopsies.
Medical College, Calcutta	2,158	127	..	86	33	3	5	1 : 2.6	1 : 17
Belgachia Medical College, Calcutta.	843	27	..	15	8	1	3	1 : 1.9	1 : 31.2
Campbell Medical School, Calcutta.	599	12	..	10	2	1 : 5	1 : 49
Total ..	3,600	166	..	111	43	4	8	1 : 2.6	1 : 21.7

TABLE II.
Showing regional distributions of cancer and incidence of malignant disease as based on autopsy records of the following institutions.

Name of institution.	CANCER.							SARCOMA.			Teratoma.	Endothelioma.	Hodgkin's disease.	Total number of malignant disease.	Total number of autopsies.
	Female genitals.	Penis.	Breast.	Gastro-intestinal tract and liver.	Buccal.	Skin.	Miscellaneous.	Rodent ulcer.	Total.	Hard.	Soft.	Total.			
Medical School, Hyderabad, Sindh.	1	..	1	1	304
B. J. M. S. Civil Hospital, Ahmedabad.	1	1	..	1	2	31
Mission Hospital, Miraj	50
Grant Medical College, Bombay.	10	8	1	81	19	3	71	..	193	3	17	20	7	224	7,823
Govardhandas Sunderdas Medical College, Bombay.	10	1	2	71	6	..	34	..	124	3	17	20	2	150	2,916
Osmania University Medical College, Hyderabad, Deccan.	1	2	3	..	2	5	50
Medical College, Calcutta	2	2	3	63	4	..	12	..	86	5	28	33	5	127	2,158
Belgachia Medical College, Calcutta.	1	12	2	..	15	2	6	8	3	27	843
Campbell Medical School, Calcutta.	2	5	3	..	10	..	2	2	..	12	599
TOTALS ..	26	11	6	235	29	3	123	..	433	13	73	86	11	548	14,774
Percentage distribution of cancer.	6.0	2.6	1.4	54.3	6.7	0.7	28.4
Percentage distribution of malignant disease.	79.0	15.7	2.0	3.0	..

TABLE III.
Incidence of malignant disease and regional distribution of cancer according to religion and sex as ascertained from post-mortem records of the pathological departments of Bombay, Bengal, small provinces, and the States.

Religion and sex.	CARCINOMA.							SARCOMA.			Teratoma.	Endothelioma.	Hodgkin's disease.	Total number of malignant disease.
	Female genitals.	Penis.	Breast.	Gastro-intestinal tract and liver.	Buccal.	Skin.	Miscellaneous.	Kidney ulcer.	Total.	Hard.	Soft.	Total.		
Hindu males	..	10	..	146	16	..	78	..	250	7	46	53	10	321
Hindu females	6	27	2	1	15	..	71	1	9	10	15	86
Mohammedan males	18	5	1	16	..	40	5	6	11	1	51
Mohammedan females	2	2	1	..	5	5
Other males	32	6	1	11	..	50	..	9	9	15	61
Other females	4	6	10	..	2	12	..	12
Unknown	..	1	..	4	2	..	7	..	1	1	1	9
TOTALS	26	11	6	235	29	3	123	..	433	13	73	86	11	545

TABLE III-a.

Incidence of malignant disease and regional distribution of cancer according to religion and sex as ascertained from post-mortem records of Bombay Presidency.

Religion and sex.	CANCER.						SARCOMA.				Hodgkin's disease.	Total number of malignant disease.			
	Female genitals.		Penis.	Breast.	Gastro-intestinal tract and liver.	CANCER.		TOTAL.	Hard.	Soft.			TOTAL.	Teratoma.	Endothelioma.
	Buccal.	Skin.				Miscellaneous.	Rodent ulcer.								
Hindu males	..	9	..	92	14	..	69	184	4	18	22	1	5	5	217
Hindu females	15	..	3	14	1	1	11	45	..	4	4	..	2	..	51
Mohammedan males	12	4	1	14	31	2	4	6	2	39
Mohammedan females	2	2	1	5	5
Christian males	25	5	..	8	38	..	7	7	2	47
Christian females	2	2	4	..	1	1	5
Anglo-Indian males	1	..	1	2	2
Anglo-Indian females	1	1	..	1	1	2
Other males	4	..	1	..	5	5
Other females	1	1	1
Unknown	1	1	2	2
TOTALS ..	20	9	3	153	25	3	105	318	6	35	41	1	7	9	376
Percentage distribution	6.2	2.8	0.9	48.1	7.8	0.9	33.0

TABLE III-b.

Incidence of malignant disease and regional distribution of cancer according to religion and sex as ascertained from post-mortem records of Bengal Presidency.

Religion and sex.	CANCER.								SARCOMA.			Total number of malignant disease.			
	Female genitals.	Penis.	Breast.	Gastro-intestinal tract and liver.	Buccal.	Skin.	Miscellaneous.	Rodent ulcer.	TOTAL.	Hard.	Soft.		Teratoma.	Endothelioma.	Hodgkin's disease.
Hindu males	1	..	52	2	..	8	..	63	3	27	30	2	5	100
Hindu females ..	4	..	3	13	1	..	4	..	25	1	4	5	1	2	33
Mohammedan males	6	1	..	2	..	9	3	2	5	1	..	15
Mohammedan females
Other males	3	2	..	5	..	2	2	7
Other females ..	1	3	4	4
Unknown	1	..	3	1	..	5	..	1	1	..	1	7
TOTALS ..	5	2	3	80	4	..	17	..	111	7	36	43	4	8	166
Percentage distribution ..	4.5	1.8	2.7	72.0	3.6	..	15.4

TABLE IV.

Showing distribution of carcinoma according to age and site and of sarcoma according to age alone as gathered from post-mortem records of Bombay, Bengal, small provinces, and the States.

Age.	Female genitals.	Penis.	Breast.	Gastro- intestinal tract and liver.	Buccal.	Skin.	Miscel- laneous.	Rodent ulcer.	TOTAL.	Sarcoma.	Tera- toma.	Endothe- lioma.	Hodgkin's disease.	TOTAL.
1-5	1	..	1	1	1	1	1	5
6-10	1	..	1	2	3
11-15	2	..	2	2	4
16-20	4	5	..	9	6	..	2	..	17
21-25 ..	1	..	1	9	3	..	7	..	21	8	..	2	4	35
26-30 ..	4	1	..	27	6	2	11	..	51	13	..	1	4	69
31-35 ..	6	1	2	25	3	..	18	..	55	11	1	67
36-40 ..	5	1	..	41	2	1	16	..	66	16	1	83
41-45 ..	5	2	2	34	3	..	9	..	55	12	2	69
46-50 ..	2	2	..	41	5	..	24	..	74	5	..	2	2	83
51-55 ..	1	..	1	16	2	..	12	..	32	2	..	3	..	37
56-60 ..	1	3	..	16	9	..	29	5	1	35
61-65	9	4	..	4	..	17	17
66-70 ..	1	4	1	..	2	..	8	8
71-75	2	2	2
76-80
81-above	1	1
Unknown	..	1	..	7	2	..	10	2	1	13

TABLE V.

Incidence of malignant disease in males as ascertained from post-mortem records given in Table I.

Name of community.	CANCER.								SARCOMA.			Total number of malignant disease.	Hodgkin's disease.	Endothelioma.	Teratoma.
	Female genitals.	Penis.	Breast.	Gastro-intestinal tract and liver.	Buccal.	Skin.	Miscellaneous.	Rodent ulcer.	Total.	Hard.	Soft.	Total.			
Hindu	10	..	146	16	..	78	..	250	7	46	53	10	7	1
Mohammedan	18	5	1	16	..	40	5	6	11	2	1	..
Others	32	6	1	11	..	50	..	9	9	2
Totals	10	..	196	27	2	105	..	340	12	61	73	14	8	1
Percentage distribution of cancer.	..	2.9	..	57.6	7.9	0.6	30.9	..	100

TABLE V-a.

Incidence of malignant disease in females as ascertained from post-mortem records given in Table I.

Name of community.	CANCER.								SARCOMA.			Teratoma.	Endothelioma.	Hodgkin's disease.	Total number of malignant disease.
	Female genitals.	Penis.	Breast.	Gastro-intestinal tract and liver.	Buccal.	Skin.	Miscellaneous.	Rodent ulcer.	TOTAL.	Hard.	Soft.	TOTAL.			
Hindu ..	20	..	6	27	2	1	15	..	71	1	9	10	3	2	86
Mohammedan ..	2	2	1	..	5	5
Others ..	4	6	10	..	2	2	12
TOTALS ..	26	..	6	35	2	1	16	..	86	1	11	12	3	2	103
Percentage distribution of cancer.	30.2	..	6.9	40.7	2.3	1.1	18.4	..	100

TABLE VI-b.
Incidence of malignant disease as based on morbid histology records of the following institutions of
Bombay Presidency.

Name of institution.	CANCER.							SARCOMA.				Total number of sections.					
	Female genitals.	Penis.	Breast.	Gastro-intestinal tract and liver.	Buccal.	Skin.	Miscellaneous.	Rodent ulcer.	Total.	Hard.	Soft.		Total.	Teratoma.	Endothelioma.	Hodgkin's disease.	Total number diagnosed as malignant disease.
Grant Medical College, Bombay.	119	72	162	43	142	16	256	15	825	29	249	278	7	32	18	1,160	6,739
Govardhandas Sunderdas Medical College, Bombay.	40	29	38	48	108	15	109	1	388	8	52	60	5	35	3	491	2,449
B. J. M. S. Civil Hospital, Ahmedabad.	1	9	12	1	2	1	17	..	43	4	22	26	..	1	1	71	..
Medical School, Poona ..	9	14	10	11	10	7	21	1	83	4	8	12	1	3	..	99	582
Mission Hospital, Miraj ..	2	1	2	1	9	..	15	2	10	12	27	85
TOTALS ..	171	125	224	103	262	40	412	17	1,354	47	341	388	13	71	22	1,848	9,855
Percentage distribution ..	12.6	9.2	16.5	7.9	19.4	2.9	30.4	1.2

TABLE VI-d.
Incidence of malignant disease as based on morbid histology records of the following institutions
in the States.

Name of institution.	CANCER.										SARCOMA.			Teratoma.	Endothelioma.	Hodgkin's disease.	Total number diagnosed as malignant disease.	Total number of sections.
	Female genitals.	Penis.	Breast.	Gastro-intestinal tract and liver.	Buccal.	Skin.	Miscellaneous.	Rodent ulcer.	Total.	Hard.	Soft.	Total.						
Medical School, Bangalore ..	2	10	3	2	1	5	8	1	32	6	15	21	53	227	
Medical College, Mysore ..	13	6	6	6	6	8	5	..	50	9	14	23	4	5	2	84	368	
Osmania University Medical College, Hyderabad Deccan.	5	1	2	2	10	..	13	13	..	4	..	27	104	
Pathological Laboratory, State General Hospital, Baroda.	2	..	3	3	13	..	21	..	3	3	1	25	..	
TOTALS ..	17	16	17	12	7	13	28	3	113	15	45	60	5	9	2	189	699	
Percentage distribution ..	15	14.1	15.0	10.6	6.2	11.5	24.8	2.6	

TABLE VII.

Incidence of malignant disease and regional distribution of cancer according to religion and sex as ascertained from histology records of the pathological departments of Bengal, Bombay, small provinces, and the States.

Religion and sex.	CANCER.								SARCOMA.			Teratoma.	Endothelioma.	Hodgkin's disease.	TOTAL.
	Female genitals.	Penis.	Breast.	Gastro-intestinal tract and liver.	Buccal.	Skin.	Miscellaneous.	Rodent ulcer.	TOTAL.	Hard.	Soft.	TOTAL.			
Hindu males	193	20	71	189	85	296	12	866	66	244	310	48	15	1,258
Hindu females ..	255	..	173	28	47	29	104	2	638	30	127	157	28	4	839
Mohammedan males.	..	4	1	22	60	23	103	6	219	24	66	90	14	11	349
Mohammedan females.	26	..	56	3	15	7	11	..	118	6	21	27	6	..	152
Other males	33	8	19	45	7	47	7	166	10	38	48	15	2	235
Other females ..	79	..	77	16	17	8	33	2	232	5	30	35	9	..	277
Unknown ..	25	7	61	19	36	11	99	10	268	15	74	89	14	8	386
TOTALS ..	385	237	396	178	409	170	693	39	2,507	156	600	756	134	40	3,496

TABLE VII-a.

Incidence of malignant disease and regional distribution of cancer according to religion and sex as ascertained from histology records of Bengal Presidency.

Religion and sex.	CANCER.									SARCOMA.			Endothelioma.	Hodgkin's disease.	Total.
	Female genitals.	Penis.	Breast.	Gastro-intestinal tract and liver.	Buccal.	Skin.	Miscellaneous.	Rodent ulcer.	Total.	Hard.	Soft.	Total.			
Hindu males	72	12	15	58	50	86	4	297	37	72	109	12	7	438
Hindu females ..	149	..	75	11	12	22	41	2	312	17	48	65	11	2	399
Mohammedan males	1	..	4	17	8	24	..	54	10	15	25	8	1	88
Mohammedan females ..	8	..	13	1	3	2	4	..	31	1	3	4	35
Other males	2	2	4	13	5	17	2	45	1	5	6	2	..	56
Other females ..	23	..	25	12	3	5	11	1	80	1	4	5	1	..	89
Unknown ..	3	4	1	1	3	6	1	1	20	2	8	10	31
TOTALS ..	183	79	128	48	109	98	184	10	839	69	155	224	34	29	1,136

TABLE VII-b.

Incidence of malignant disease and regional distribution of cancer according to religion and sex as ascertained from histology records of Bombay Presidency.

Religion and sex.	CANCER.								SARCOMA.			Teratoma.	Endothelioma.	Hodgkin's disease.	TOTAL.	
	Female genitals.	Penis.	Breast.	Gastro-intestinal tract and liver.	Buccal.	Skin.	Miscellaneous.	Rodent ulcer.	TOTAL.	Hard.	Soft.					TOTAL.
Hindu males	94	6	44	117	23	173	8	465	19	131	150	4	27	5	651
Hindu females ..	86	..	78	14	28	3	44	..	253	8	56	64	..	10	1	328
M o h a m m e d a n males.	..	3	..	14	35	9	58	2	121	7	31	38	2	6	7	174
M o h a m m e d a n females.	15	..	34	1	11	..	5	..	66	2	13	15	1	2	..	84
Other males	28	5	12	31	..	27	3	106	4	33	37	..	10	1	154
Other females ..	52	..	48	4	12	1	21	..	138	1	22	23	..	5	..	166
Unknown ..	18	..	53	14	28	4	84	4	205	6	55	61	6	11	8	291
TOTALS ..	171	125	224	103	262	40	412	17	1,354	47	341	388	13	71	22	1,848

TABLE VII-c.

Incidence of malignant disease and regional distribution of cancer according to religion and sex as ascertained from histology records of small provinces.

Religion and sex.	CANCER.								SARCOMA.			Teratoma.	Endothelioma.	Hodgkin's disease.	Total.	
	Female genitals.	Penis.	Breast.	Gastro-intestinal tract and liver.	Buccal.	Skin.	Miscellaneous.	Rodent ulcer.	Total.	Hard.	Soft.					Total.
Hindu males	13	1	4	11	5	24	..	58	24	1	5	90	
Hindu females ..	5	..	11	2	3	1	8	..	30	11	..	8	50	
Mohammedan males	3	8	5	20	4	40	21	3	6	72	
Mohammedan females	3	..	5	1	1	4	2	..	10	5	..	3	24	
Other males	1	2	1	2	2	2	12	5	2	1	21	
Other females ..	3	3	4	..	2	2	1	1	13	7	..	1	21	
Unknown ..	3	2	5	3	5	..	12	2	32	11	1	1	45	
TOTALS ..	14	17	27	15	31	19	69	9	201	84	7	25	323	

TABLE VII-d.

Incidence of malignant disease and regional distribution of cancer according to religion and sex as ascertained from histology records of the States.

Religion and sex.	CANCER.								SARCOMA.			Hodgkin's disease.	Total.
	Female genitals.	Penis.	Breast.	Gastro-intestinal tract and liver.	Buccal.	Skin.	Miscellaneous.	Rodent ulcer.	Total.	Hard.	Soft.	Total.	
Hindu males	14	1	8	3	7	13	..	46	9	18	27	79
Hindu females ..	15	..	9	1	4	3	11	..	43	5	12	17	62
Mohammedan males	1	1	..	1	1	..	4	..	6	6	15
Mohammedan females	4	1	..	5	..	3	3	9
Other males	1	..	1	1	..	3	4
Other females ..	1	1	1
Unknown ..	1	1	2	1	..	2	1	3	11	1	6	7	19
TOTALS ..	17	16	17	12	7	13	28	3	113	15	45	60	189

TABLE VIII.

Regional distribution of cancer per hundred male cases based on the records of the pathological laboratories of Bengal, Bombay, small provinces, and the States.

Religion.	CANCER.							
	Female genitals.	Penis.	Breast.	Gastro-intestinal tract and liver.	Buccal.	Skin.	Miscellaneous.	Rodent ulcer.
		22.2	2.3	8.2	21.8	9.8	34.1	1.4
Hindu	1.8	0.45	10.0	27.4	10.4	47.0	2.7
Mohammedan	19.8	4.8	11.4	27.1	4.2	28.3	4.2
Others							
Combined Hindu, Mohammedan and others.	..	18.4	2.3	8.9	23.5	9.2	35.6	1.9

TABLE VIII-a.

Regional distribution of cancer per hundred male cases based on the records of the pathological laboratories of Bengal Presidency.

Religion.	Female genitals.	Penis.	Breast.	Gastro-intestinal tract and liver.	Buccal.	Skin.	Miscellaneous.	Rodent ulcer.
Hindu	24.2	4.2	5.4	19.5	16.6	28.6	1.3
Mohammedan	1.8	..	7.4	31.5	14.8	43.4	..
Others	4.4	4.4	4.8	28.8	11.1	37.7	4.4
Combined Hindu, Mohammedan and others.	..	18.9	3.5	5.6	22.0	16.0	32.0	1.5

TABLE VIII-b.

Regional distribution of cancer per hundred male cases based on the records of the pathological laboratories of Bombay Presidency.

Religion.	Female genitals.	Penis.	Breast.	Gastro- intestinal tract and liver.	Buccal.	Skin.	Miscel- laneous.	Rodent ulcer.
Hindu	20·2	1·3	9·4	25·1	4·9	37·2	1·7
Mohammedan	2·5	..	11·5	28·9	7·5	47·9	1·6
Others	26·4	4·7	11·3	29·2	..	25·4	2·9
Combined Hindu, Mohammedan and others.	..	18·0	1·6	10·2	26·5	4·7	35·9	1·9

TABLE VIII-c.

Regional distribution of cancer per hundred male cases based on the records of the pathological laboratories of small provinces.

Religion.	Female genitals.	Penis.	Breast.	Gastro- intestinal tract and liver.	Buccal.	Skin.	Miscel- laneous.	Rodent ulcer.
Hindu	22·4	1·7	6·9	18·9	8·6	41·4	..
Mohammedan	7·5	20·0	12·5	50·0	10·0
Others	16·6	8·3	16·6	8·3	16·6	16·6	16·6
Combined Hindu, Mohammedan and others.	..	13·6	1·8	8·1	18·1	10·9	41·8	54·5

TABLE VIII-d.

Regional distribution of cancer per hundred male cases based on the records of the pathological laboratories of the States.

Religion.	Female genitals.	Penis.	Breast.	Gastro- intestinal tract and liver.	Buccal.	Skin.	Miscel- laneous.	Rodent ulcer.
Hindu	30.4	2.2	17.4	6.5	15.2	28.3	..
Mohammedan	25.0	25.0	..	25.0	25.0	..
Others	33.0	..	33.0	33.0	..
Combined Hindu, Mohammedan and others.	..	28.3	3.8	18.8	5.7	15.0	28.3	..

TABLE IX.

Regional distribution of cancer per hundred female cases based on the records of the pathological laboratories of Bengal, Bombay, small provinces, and the States.

Religion.	CANCER.							
	Female genitals.	Penis.	Breast.	Gastro- intestinal tract and liver.	Buccal.	Skin.	Miscel- laneous.	Rodent ulcer.
Hindu ..	39.9	..	27.1	4.3	7.3	4.5	16.3	0.32
Mohammedan ..	22.0	..	47.4	2.5	12.7	5.9	9.3	..
Others ..	33.6	..	33.2	6.9	7.3	3.5	14.2	0.8
Combined Hindu, Mohammedan and others.	36.4	..	30.9	4.7	8.0	4.4	14.95	0.42

TABLE VIII-b.

Regional distribution of cancer per hundred male cases based on the records of the pathological laboratories of Bombay Presidency.

Religion.	Female genitals.	Penis.	Breast.	Gastro- intestinal tract and liver.	Buccal.	Skin.	Miscel- laneous.	Rodent ulcer.
Hindu	20.2	1.3	9.4	25.1	4.9	37.2	1.7
Mohammedan	2.5	..	11.5	28.9	7.5	47.9	1.6
Others	26.4	4.7	11.3	29.2	..	25.4	2.9
Combined Hindu, Mohammedan and others.	..	18.0	1.6	10.2	26.5	4.7	35.9	1.9

TABLE VIII-c.

Regional distribution of cancer per hundred male cases based on the records of the pathological laboratories of small provinces.

Religion.	Female genitals.	Penis.	Breast.	Gastro- intestinal tract and liver.	Buccal.	Skin.	Miscel- laneous.	Rodent ulcer.
Hindu	22.4	1.7	6.9	18.9	8.6	41.4	..
Mohammedan	7.5	20.0	12.5	50.0	10.0
Others	16.6	8.3	16.6	8.3	16.6	16.6	16.6
Combined Hindu, Mohammedan and others.	..	13.6	1.8	8.1	18.1	10.9	41.8	54.5

TABLE IX-c.

Regional distribution of cancer per hundred female cases based on the records of the pathological laboratories of small provinces.

Religion.	Female genitals.	Penis.	Breast.	Gastro- intestinal tract and liver.	Buccal.	Skin.	Miscel- laneous.	Rodent ulcer.
Hindu ..	16.6	..	36.6	6.6	10.0	3.3	26.6	..
Mohammedan ..	18.7	..	31.2	6.2	6.2	25.0	12.5	..
Others ..	23.6	..	30.7	..	15.4	15.4	7.7	7.1
Combined Hindu, Mohammedan and others.	19.5	..	33.9	5.1	10.2	11.9	18.7	1.9

TABLE IX-d.

Regional distribution of cancer per hundred female cases based on the records of the pathological laboratories of the States.

Religion.	Female genitals.	Penis.	Breast.	Gastro- intestinal tract and liver.	Buccal.	Skin.	Miscel- laneous.	Rodent ulcer.
Hindu ..	34.8	..	20.9	2.3	9.3	6.9	25.6	..
Mohammedan	80.0	20.0	..
Others ..	100.0
Combined Hindu, Mohammedan and others.	32.1	..	26.1	2.0	8.0	6.0	24.0	..

TABLE X.

Showing distribution of carcinoma according to age and site and of sarcoma according to age alone as gathered from pathological histology records of Bengal, Bombay, small provinces, and the States.

Age.	Female genitals.	Penis.	Breast.	Gastro-intestinal tract and liver.	Buccal.	Skin.	Miscellaneous.	Rodent ulcer.	Total.	Sarcoma.	Teratoma.	Endothelioma.	Hodgkin's disease.	Total.
1-5	1	..	1	16	2	6	..	25
6-10	2	4	..	6	15	4	2	6	33
11-15	1	1	2	1	2	..	7	16	1	3	1	28
16-20	6	2	7	5	6	..	27	31	5	9	1	73
21-25	15	2	1	6	11	4	23	..	67	29	9	10	1	116
26-30	27	13	9	11	24	10	24	..	118	30	7	5	1	160
31-35	34	20	26	11	21	18	37	..	167	37	6	10	3	223
36-40	40	17	36	18	38	12	47	1	209	32	2	8	..	251
41-45	29	17	39	20	22	15	38	1	181	30	1	7	1	220
46-50	31	15	30	18	39	13	46	1	193	24	2	4	1	224
51-55	12	7	16	8	16	8	32	1	100	17	1	2	1	121
56-60	8	16	9	11	26	7	26	2	105	12	..	4	..	121
61-65	5	5	6	3	9	3	10	..	41	41
66-70	2	2	2	1	6	2	11	3	29	29
71-75	1	2	..	2	6	..	4	..	15	1	16
76-80	2	1	1	3	3	..	10	10
81-above	215	68	1	1	..	1	3	3
Unknown	170	118	181	68	379	29	1,228	466	19	64	25	1,802
TOTALS ..	385	237	396	178	409	170	693	39	2,507	756	59	134	40	3,496

TABLE XI-c.

Incidence of malignant disease as based on hospital records of the following institutions in small provinces.

Name of the institution.	CANCER.								SARCOMA.			Teratoma.	Endothelioma.	Hodgkin's disease.	Total number diagnosed as malignant disease.	Total number of cases treated.
	Female genitals.	Penis.	Breast.	Gastro-intestinal tract and liver.	Buccal.	Skin.	Miscellaneous.	Rodent ulcer.	TOTAL.	Hard.	Soft.	TOTAL.				
Welsh Missionary Hospital, Shillong.	9	1	2	11	20	9	11	1	64	2	10	12	1	..	77	11,370
Dibrugarh Hospital ..	8	7	3	11	4	9	6	1	49	5	16	21	70	4,608
Civil Hospital, Nagpur ..	19	7	17	22	10	7	27	..	109	8	12	20	..	1	132	11,815
Medical School Hospital, Hyderabad Sindh.	18	11	36	48	24	22	45	6	210	4	34	38	..	5	259	12,574
Civil Hospital, Karachi ..	2	..	5	12	2	..	7	..	28	..	4	4	32	5,822
TOTALS ..	56	26	63	104	60	47	96	8	460	19	76	95	1	7	570	46,189
Percentage distribution ..	12.2	5.6	13.7	22.6	13.05	10.2	20.8	1.7

TABLE XI-d.

Incidence of malignant disease as based on hospital records of the following institutions in the States.

Incidene of malignant disease as disea on hospita reeords of malyeary instiue

Name of the institution.	CANCER.								SARCOMA.			Teratoma.	Endothelioma.	Hodgkin's disease.	Total number diagnosed as malignant disease.	Total number of cases treated.	
	Female genitals.	Penis.	Breast.	Gastro-intestinal tract and liver.	Buccal.	Skin.	Miscellaneous.	Rodent ulcer.	Total.	Hard.	Soft.						Total.
Medical School Hospital, Bangalore.	13	12	10	29	8	5	11	1	89	7	11	18	1	1	109	15,119	
Medical College Hospital, Mysore.	13	13	6	3	24	3	13	2	77	..	7	7	1	..	88	16,669	
Osmania University Medical Hospital, Hyderabad Deccan.	24	6	38	22	53	5	12	..	160	2	46	48	208	..	
King Edward Medical Hospital, Indore.	22	18	27	34	70	7	27	..	205	4	22	26	..	3	238	10,565	
Civil Hospital, Udaipur	2	3	2	2	3	1	..	13	..	1	1	14	1,050	
Civil Hospital, Ajmer ..	1	1	3	1	3	..	3	..	12	..	6	6	..	1	20	1,832	
State General Hospital, Baroda.	57	20	43	9	515	15	219	..	878	14	49	63	941	..	
Gopaljee Valjee Radium Institute, Jammagar.	14	5	11	1	43	5	1	1	81	1	3	4	85	..	
TOTALS ..	144	77	141	101	718	43	287	4	1,515	28	145	173	2	5	1,703	45,238	
Percentage distribution ..	9.4	5.1	9.3	6.9	47.3	2.8	18.9	0.3	

TABLE XII.

Combined table showing cases of malignant disease according to sex, religion, and site in Bombay, Bengal, small provinces, and the States.

Religion and sex.	CANCER.						SARCOMA.			Teratoma.	Endothelioma.	Hodgkin's disease.	Total.
	Female genitals.	Penis.	Breast.	Gastro-intestinal tract and liver.	Buccal.	Skin.	Miscellaneous.	Rodent ulcer.	Total.	Hard.	Soft.	Total.	
Hindu males	342	27	611	947	134	744	18	2,823	183	293	476	3,353
Hindu females ..	682	..	392	228	177	60	207	2	1,748	60	115	175	1,943
Mohammedan males.	..	15	4	239	262	59	242	6	827	54	119	173	1,035
Mohammedan females.	102	..	118	45	76	9	36	1	387	12	24	36	426
Other males	15	1	96	80	9	70	5	276	9	27	36	318
Other females ..	138	..	102	62	29	23	32	4	390	6	27	33	427
Unknown ..	4	7	7	5	11	1	35	4	6	10	68
TOTALS ..	926	372	644	1,288	1,578	299	1,342	37	6,486	328	611	939	7,570

TABLE XII-a.

Combined table showing cases of malignant disease according to sex, religion, and site in the institutions of Bombay Presidency.

of Bombay and Hyderabad.

Religion and sex.	CANCER.							SARCOMA.				Hodgkin's disease.	Total.			
	Female genitals.	Penis.	Breast.	Gastro-intestinal tract and liver.	Buccal.	Skin.	Miscellaneous.	Rodent ulcer.	Total.	Hard.	Soft.			Tetatomia.	Endothelioma.	
Hindu males	126	6	267	297	38	282	13	1,029	43	94	137	3	7	16	1,192
Hindu females ..	287	..	136	75	62	7	69	2	638	25	36	61	1	6	5	711
M o h a m m e d a n males.	..	2	..	104	139	7	97	2	351	12	25	37	3	6	7	404
M o h a m m e d a n females.	43	..	27	19	28	1	13	..	131	2	4	6	1	138
Other males	6	1	68	51	1	42	2	171	6	15	21	1	1	2	196
Other females ..	75	..	55	28	24	1	20	4	207	1	13	14	..	3	..	221
Unknown ..	4	6	6	1	9	..	26	2	3	5	31
TOTALS ..	409	134	225	567	607	56	532	23	2,553	91	190	281	8	23	31	2,896

TABLE XII-b.

Combined table showing cases of malignant disease according to sex, religion, and site in the institutions of Bengal Presidency.

Religion and sex.	CANCER.								SARCOMA.			Teratoma.	Endothelioma.	Hodgkin's disease.	TOTAL.
	Female genitals.	Penis.	Breast.	Gastro-intestinal tract and liver.	Buccal.	Skin.	Miscellaneous.	Rodent ulcer.	TOTAL.	Hard.	Soft.	TOTAL.			
Hindu males	122	16	255	96	50	220	2	761	112	90	202	7	4	975
Hindu females ..	239	..	131	119	38	45	93	..	665	28	45	73	1	3	743
Mohammedan males.	..	6	3	78	34	34	76	..	231	36	45	81	6	3	321
Mohammedan females.	33	..	25	15	12	5	10	..	100	6	4	10	1	1	112
Other males	7	..	20	11	..	20	..	58	2	6	8	..	1	67
Other females ..	45	..	40	29	1	18	7	..	140	5	10	15	1	..	156
Unknown	1	1	1	..	3	1	..	1	..	23	27
TOTALS ..	317	135	215	516	193	153	427	2	1,958	190	200	390	16	35	2,401

TABLE XII-c.

Combined table showing cases of malignant disease according to sex, religion, and site in the institutions of small provinces.

Religion and sex.	CANCER.										SARCOMA.		Teratoma.	Endothelioma.	Hodgkin's disease.	Total.
	Female genitals.	Penis.	Breast.	Gastro-intestinal tract and liver.	Rectum.	Skin.	Miscellaneous.	Rodent ulcer.	Total.	Hard.	Soft.	Total.				
Hindu males	20	2	41	13	14	36	1	127	10	31	41	172
Hindu females ..	35	..	29	14	6	3	11	..	98	4	7	11	..	3	..	112
Mohammedan males	4	1	38	19	17	35	4	118	3	21	24	5	149
Mohammedan females	13	..	29	2	5	1	4	1	55	1	5	6	61
Other males	2	..	3	13	8	5	1	32	..	6	6	1	39
Other females ..	8	..	2	5	4	1	4	..	24	..	4	4	28
Unknown	1	..	3	1	1	6	1	2	3	9
TOTALS ..	56	26	63	104	60	47	96	8	460	19	76	95	1	7	7	570

TABLE XII-d.

Combined table showing cases of malignant disease according to sex, religion, and site in the institutions of the States.

Religion and sex.	CANCER.								SARCOMA.			Teratoma.	Endothelioma.	Hodgkin's disease.	TOTAL.
	Female genitals.	Penis.	Breast.	Gastro-intestinal tract and liver.	Buccal.	Skin.	Miscellaneous.	Rodent ulcer.	TOTAL.	Hard.	Soft.	TOTAL.			
Hindu males	74	3	48	542	32	206	2	907	18	78	96	4	5	1,014
Hindu females ..	121	..	96	20	71	5	34	..	347	3	27	30	377
Mohammedan males.	..	3	..	19	70	1	34	..	127	3	28	31	1	3	162
Mohammedan females.	13	..	37	9	30	2	9	..	100	3	11	14	114
Other males	5	5	..	3	2	15	1	..	1	16
Other females ..	10	..	5	3	1	..	19	19
Unknown	1	1	1
TOTALS ..	144	77	141	101	718	43	287	4	1,515	28	145	173	5	8	1,703

TABLE XIII.

Regional distribution of cancer per hundred male cases according to communities as ascertained from the records of hospitals in Bombay, Bengal, small provinces, and the States.

Name of community.	Female genitals.	Penis.	Breast.	Gastro-intestinal tract and liver.	Buccal.	Skin.	Miscellaneous.	Rodent ulcer.
Hindu	12.1	0.9	21.6	33.5	4.7	26.3	0.6
Mohammedan	1.8	0.4	28.9	31.6	7.1	29.2	0.7
Others	5.4	0.3	34.7	28.9	3.2	25.3	1.8
Combined communities.	..	9.5	0.8	24.0	32.8	5.1	26.9	0.75

TABLE XIII-a.

Regional distribution of cancer per hundred male cases according to communities in Bombay Presidency.

Name of community.	Female genitals.	Penis.	Breast.	Gastro-intestinal tract and liver.	Buccal.	Skin.	Miscellaneous.	Rodent ulcer.
Hindu	12.2	0.58	25.9	28.8	3.7	27.4	1.2
Mohammedan	0.6	..	29.6	39.6	1.95	27.6	0.6
Others	3.5	0.6	39.7	29.8	0.6	24.7	1.2
Combined communities.	..	9.0	0.9	30.1	33.5	3.1	22.1	1.2

TABLE XIII-b.

Regional distribution of cancer per hundred male cases according to communities in Bengal Presidency.

Name of community.	Female genitals.	Penis.	Breast.	Gastro-intestinal tract and liver.	Buccal.	Skin.	Miscellaneous.	Rodent ulcer.
Hindu	16.1	2.1	33.5	12.6	6.5	28.9	0.26
Mohammedan	2.5	1.3	33.8	14.7	14.7	32.9	..
Others	12.0	..	34.5	18.9	..	34.5	..
Combined communities.	..	12.8	1.8	33.5	13.4	8.0	30.0	0.19

TABLE XIII-c.

Regional distribution of cancer per hundred male cases according to communities in small provinces.

Name of community.	Female genitals.	Penis.	Breast.	Gastro-intestinal tract and liver.	Buccal.	Skin.	Miscellaneous.	Rodent ulcer.
Hindu	15.7	1.5	31.8	10.2	11.0	28.3	0.8
Mohammedan	3.3	0.85	31.9	15.9	14.3	29.4	3.3
Others	6.4	..	9.7	41.9	25.8	16.1	3.2
Combined communities.	..	9.4	1.08	29.7	16.2	14.0	27.1	2.2

TABLE XIII-d.

Regional distribution of cancer per hundred male cases according to communities in the States.

Name of community.	Female genitals.	Penis.	Breast.	Gastro-intestinal tract and liver.	Buccal.	Skin.	Miscellaneous.	Rodent ulcer.
Hindu	8.1	0.3	5.2	67.6	3.5	22.6	0.2
Mohammedan	2.3	..	14.9	55.1	0.8	26.7	..
Others	33.3	33.3	..	20.0	13.3
Combined communities.	..	7.3	0.3	6.0	65.5	3.1	23.1	0.4

TABLE XIV.

Regional distribution of cancer per hundred female cases according to communities as ascertained from the records of hospitals in Bombay, Bengal, small provinces, and the States.

Name of community.	Female genitals.	Penis.	Breast.	Gastro-intestinal tract and liver.	Buccal.	Skin.	Miscellaneous.	Rodent ulcer.
Hindu	39.0	..	22.4	13.0	10.1	3.4	11.7	0.1
Mohammedan ..	26.3	..	30.5	11.6	19.6	2.3	9.3	0.2
Others	35.4	..	26.1	15.9	7.4	5.9	8.3	1.0
Combined communities.	36.5	..	24.2	13.2	12.2	3.6	10.8	0.27

TABLE XIV-a.

Regional distribution of cancer per hundred female cases according to communities as ascertained from the records of hospitals in Bombay Presidency.

Name of community.	Female genitals.	Penis.	Breast.	Gastro-intestinal tract and liver.	Buccal.	Skin.	Miscellaneous.	Rodent ulcer.
Hindu ..	43.5	..	21.3	11.7	9.7	1.1	10.6	0.3
Mohammedan ..	32.7	..	20.6	14.5	21.3	0.7	9.9	..
Others ..	36.2	..	26.5	13.5	11.6	04.9	9.6	1.9
Combined communities.	41.6	..	22.3	12.5	11.7	0.9	10.4	0.6

TABLE XIV-b.

Regional distribution of cancer per hundred female cases according to communities as ascertained from the records of hospitals in Bengal Presidency.

Name of community.	Female genitals.	Penis.	Breast.	Gastro-intestinal tract and liver.	Buccal.	Skin.	Miscellaneous.	Rodent ulcer.
Hindu ..	36.0	..	19.7	17.9	5.7	6.8	13.9	..
Mohammedan ..	32.6	..	24.7	14.8	12.8	4.9	9.9	..
Others ..	32.1	..	28.6	20.7	0.7	12.8	5.0	..
Combined communities.	34.9	..	21.6	17.9	5.7	7.5	11.1	.

TABLE XIV-c.

Regional distribution of cancer per hundred female cases according to communities as ascertained from the records of hospitals in small provinces.

Name of community.	Female genitals.	Penis.	Breast.	Gastro-intestinal tract and liver.	Buccal.	Skin.	Miscellaneous.	Rodent ulcer.
Hindu ..	35.7	..	29.4	11.3	6.2	3.1	11.2	..
Mohammedan ..	23.6	..	52.7	3.6	9.0	1.8	7.2	1.8
Others ..	33.3	..	8.3	20.8	16.6	4.1	16.6	..
C o m b i n e d communities.	32.6	..	33.4	11.7	8.4	2.8	10.7	0.5

TABLE XIV-d.

Regional distribution of cancer per hundred female cases according to communities as ascertained from the records of hospitals in the States.

Name of community.	Female genitals.	Penis.	Breast.	Gastro-intestinal tract and liver.	Buccal.	Skin.	Miscellaneous.	Rodent ulcer.
Hindu ..	34.9	..	27.7	5.7	20.5	1.4	9.5	..
Mohammedan ..	13.0	..	37.0	9.0	30.0	2.0	9.0	..
Others ..	52.6	..	26.3	15.6	5.2	..
C o m b i n e d communities.	30.9	..	29.6	6.2	21.7	2.1	9.2	..

TABLE XV.

Showing distribution of carcinoma according to age and site and of sarcoma according to age alone as gathered from hospital records of Bombay, Bengal, small provinces, and the States.

Age.	Female genitals.	Penis.	Breast.	Gastro-intestinal tract and liver.	Buccal.	Skin.	Miscellaneous.	Rodent ulcer.	Total.	Sarcoma.	Teratoma.	Endothelioma.	Hodgkin's disease.	Total.
1-5	2	2	7	..	11	31	1	43
6-10	3	..	1	..	4	2	6	..	16	39	7	63
11-15	2	3	2	1	4	..	12	46	..	1	4	63
16-20	15	..	6	9	8	9	20	..	67	96	..	1	7	175
21-25	38	10	9	35	27	14	60	3	196	110	5	4	13	328
26-30	104	38	43	86	62	23	105	..	461	115	7	9	9	598
31-35	106	43	61	127	105	35	146	1	624	105	6	6	6	745
36-40	174	50	129	213	201	47	147	4	965	95	1	8	5	1,071
41-45	135	43	102	180	142	28	141	3	774	79	2	4	2	864
46-50	125	55	113	242	191	40	191	9	966	63	3	2	2	1,030
51-55	54	29	38	125	94	27	83	6	456	36	2	1	..	495
56-60	64	45	64	131	127	30	107	6	574	32	..	1	..	609
61-65	22	18	16	65	40	10	42	..	213	6	1	219
66-70	9	8	12	27	32	6	28	..	122	5	128
71-75	3	7	3	16	14	1	13	2	59	3	62
76-80	3	2	2	3	1	2	9	1	23	2	25
81-above	1	2	..	2	1	..	3	1	10	2	12
Unknown	68	22	45	24	525	22	230	1	937	74	23	1,034
Totals ..	926	372	644	1,288	1,578	299	1,342	37	6,486	939	27	37	81	7,570

COMPLEMENT-FIXATION IN LEPROSY AND OTHER
DISEASES BY THE WITEBSKY, KLINGENSTEIN
AND KUHN (W. K. K.) ANTIGEN.

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INTRODUCTION.

It is over thirty years since the first attempt was made to establish a satisfactory complement-fixation test for use in diagnosis of leprosy, the first publication on the subject being that of Eitner (1906). Eitner used as antigen in two cases an aqueous extract of leproma. For the next ten years much attention was paid to the subject by many workers, but a satisfactory test was not forthcoming and interest then appeared to slacken. Recently, however, interest in the subject has revived particularly in view of the published results of the test performed with the W. K. K. antigen.

It is unlikely that a complement-fixation test will be of great practical value in the diagnosis of leprosy, since leprosy is a disease which shows itself outwardly at an early stage, and in which early clinical diagnosis presents few difficulties to the experienced worker. It is possible, however, that complement-fixation tests may be of value in prognosis, in assessing results of treatment, and in testing the antigenic properties of supposed cultures of the organism.

THE LITERATURE OF COMPLEMENT-FIXATION IN LEPROSY.

The early literature on the subject is very ably reviewed by Cooke (1919) and to cover this period we shall summarize his article.

He reviews and tabulates the results reported by many workers. He summarizes in tabular form the results of complement-fixation tests of various kinds carried out with various antigens. Cooke's Table I refers to the Wassermann reaction and deals with 42 reports. The aggregate of the reported results is as follows:—

Sera tested	1,397
Percentage positive	50
Nodular and mixed cases	723
Percentage positive	60
Anæsthetic cases	405
Percentage positive	25

Cooke's Table II refers to complement-fixation with alcoholic extracts of lepromas, sarcomas, carcinomas, and normal skin as antigen. Eight workers are quoted with aggregate results as follows:—

Sera tested	210
Percentage positive	52
Nodular and mixed cases	109
Percentage positive	75
Anæsthetic cases	91
Percentage positive	25

Cooke's Table III refers to complement-fixation with antigens prepared from lepromas by aqueous extraction. Sixteen workers are quoted with the following aggregate results:—

Sera tested	308
Percentage positive	66
Nodular and mixed cases	144
Percentage positive	84
Anæsthetic cases	137
Percentage positive	41

Cooke's Table IV refers to complement-fixation with bacterial antigen (tuberculin). Nine workers are quoted with the following results:—

Sera tested	180
Percentage positive	68
Nodular and mixed cases	44
Percentage positive	84
Anæsthetic cases	17
Percentage positive	35

Cooke's Table V refers to complement-fixation with bacterial antigens (emulsions of various acid-fast bacilli, chiefly tubercle bacilli or supposed leprosy cultures). Eight workers are quoted with the following aggregate results:—

Sera tested	89
Percentage positive	83
Nodular and mixed cases	55
Percentage positive	93
Anæsthetic cases	12
Percentage positive	58

In all this work Cooke notes the apparent lack of specificity of the reaction (antigens prepared from non-leprous material frequently giving positive results), and also the failure of most workers to do adequate control tests.

Cooke then gives details of his own work on complement-fixation in fifteen cases of leprosy using as antigens nineteen different bacillary emulsions, including emulsions made from tubercle bacilli and from bacilli from supposed leprosy cultures. He concludes that leprous sera contain a complement-binding substance varying very much in amount, the nodular cases showing the highest amount of the substance. The substance acts with acid-fast bacilli of various kinds, and also sometimes with 'non-acid-fast' antigens but only in low dilution. This group reaction given by leprous sera with many acid-fast bacilli, prevents the use of complement-fixation tests to prove that any particular acid-fast organism isolated from leprous lesions is really the organism of leprosy.

Since the time of Cooke's publication, the attention of various workers has been directed towards the preparation of a better antigen and towards the improvement of technique so as to render complement-fixation tests more specific.

Lewis and Aronson (1923) used an alcoholic extract of bovine tubercle bacilli, and in 43 cases of all types obtained 97.7 per cent of positive results, but of 152 control sera, 31.6 per cent gave positive results.

Taylor and Malone (1924) used an antigen prepared from *B. tuberculosis* by de-fatting with formalin and acetone according to the technique of Dreyer, standardizing the antigen by means of opacity tests. One hundred tests were performed with the following results :—

				Per cent positive.
Nodular 37 cases	100
Mixed 13 cases	92
Anæsthetic 50 cases	96

Fourteen non-leprosy, non-tuberculous, and Wassermann negative sera gave all negative results.

Raevsky and Braoul (1926) used an antigen prepared from the tubercle bacillus. They obtained positive results in 39 out of 44 nodular cases. In slight and inactive cases the percentage of positive results was much lower.

Row (1937) briefly mentions the use of antigen prepared by autolysis of tubercle bacilli, with 100 per cent positive results in cases of 'cutaneous' type, 100 per cent negative results in cases of neural type, and doubtful results in cases of 'tuberculoid' type. No details have been published.

Considerable attention has recently been paid to the use of the antigen prepared from tubercle bacilli by the Witebsky, Klingenstein and Kuhn (1931) method. In 1931 these authors published their method of preparation and since then the antigen has been widely used in tuberculosis and is handled commercially by Bayer. It is commonly called the W. K. K. antigen.

Brants (1932) reported on the use of this antigen in leprosy, reporting positive findings in 100 per cent of cases of nodular type, and in 79 per cent of cases of neural type.

Kornel (1933) used W. K. K. antigen in cases of leprosy, suspected leprosy, contacts with leprosy, and cases of tuberculosis and syphilis.

Of 14 cases of leprosy 13 gave positive results.

Of 5 suspects and contacts 2 gave positive results.

Of 10 cases of tuberculosis 6 gave positive results.

Of 10 cases of syphilis 6 gave positive results.

Using as antigen an alcoholic extract of leproma, Kornel obtained similar results except that few positive findings were made in tuberculous sera.

Bier and Arnold (1935) reported the following results from the use of W. K. K. antigen :—

				Number of cases.	Number positive.	Per cent positive.
Neural leprosy		14	8	57.1
Nodular		31	29	93.5
Mixed		155	146	94.2
Incipient cases		72	32	44.4
TOTALS	..			272	215	79

Bier (1936) reported 94 per cent of nodular and mixed cases positive and 70 per cent of neural cases positive. Leishmaniasis showed 70 per cent positive.

Rabello and Machado (1936) report 26 per cent of positive findings in cases of tuberculoid leprosy.

Pereira (1936) gives the following results :—

	Number.	— per cent.	± per cent.	+	++	+++	Per cent positive.
				per cent.	per cent.	per cent.	
Skin leprosy ..	12	0	0	0	17	83	100
Mixed „ ..	35	0	0	0	31	69	100
Nerve „ ..	60	15	5	13	47	20	80
Contacts ..	84	63	5	11	15	6	32
Syphilis ..	3	100	<i>Nil.</i>
Healthy ..	8	100	<i>Nil.</i>

Rabello (1937) writing on classification makes the following remarks about the results of the Witebsky test in the different types of leprosy :—

	Per cent
Lepromatous type ..	90 positive strong.
Anæsthetic type ..	60 „ „
Macular type ..	20 „ „
Tuberculoid type ..	80 negative.

Aoki and Murao (1933) prepared an antigen from leprous nodules, by a method similar to that of Witebsky, Klingenstein and Kuhn, and performed the complement-fixation test in a series of cases of nodular and nerve leprosy, tuberculosis, and syphilis, and in healthy controls, and on some of the cases performed the original W. K. K. test also. With the leprosy antigen, the results obtained were similar to, but not so definite as, those obtained by the use of W. K. K. antigen, most cases of leprosy, and some cases of tuberculosis and of syphilis giving positive results, healthy controls giving negative results.

This review of the literature indicates that the best available antigen for complement-fixation in leprosy appears to be an antigen prepared from the tubercle bacillus, and that the most promising results have been reported from the use of the antigen of Taylor and Malone, and of Witebsky, Klingenstein and Kuhn. The latter antigen has been used in many cases by several workers and is on the market, whereas the other antigen has been used only by its originators and is not available. We have, therefore, undertaken a study of the use of the W. K. K. antigen.

Two different types of result have been reported from the use of W. K. K. antigen. All workers report a very high percentage of positive findings in cases of lepromatous (nodular, cutaneous, or skin) type. Some workers report also a moderately high percentage of positive findings in cases of neural type, while others report that in this type of leprosy, the result is usually negative. It is possible, even probable, that this divergence of opinion may to some extent be caused by differences in methods of classification.

In the system of classification of cases laid down by the Manila Conference and recently modified by the Cairo Conference, a case is primarily classified as 'lepromatous' when any lesion of this type is present even if there are also marked lesions of the nerves; and a case is only classified as 'neural' when *all* the lesions present are of this type. Possibly some workers have applied this system rigidly, while others

have not, but have classified a case as 'neural' if the predominating symptoms were anæsthesia, deformities, etc., ignoring or perhaps overlooking the presence of slight lepromatous lesions. As will be shown later in this paper, we find that the presence of even slight lesions of this type influences markedly the results of complement-fixation tests.

TECHNIQUE OF THE TEST AND READING OF THE RESULTS.

We have elsewhere described in detail our method of using W. K. K. antigen in complement-fixation in leprosy (Greval *et al.*, 1939.) Our technique differs markedly from the technique described by Witebsky, Klingenstein and Kuhn, which involves the use of seven dilutions of serum for the test proper. In our method the test proper is done in one tube. In spite of its greater simplicity, the sensitivity of our test is at least equal to that of the test described by Witebsky, Klingenstein and Kuhn. A parallel series of tests of 50 sera by both techniques gave practically identical results. Since we are using only one tube instead of seven, the readings of the two techniques are not strictly comparable. We have recorded our results in one tube as follows:—

Inhibition complete, no hæmolysis	.. +	corresponding to	+++	in original test.
Inhibition almost complete, traces of hæmolysis.	.. T	"	++	" " "
Inhibition partial, hæmolysis	.. ±	"	+	" " "
No inhibition, complete hæmolysis	.. —	"	—	" " "

RESULTS OF THE TEST.

The results of the test in 250 cases of leprosy, the cases being divided into three groups according to the results of the clinical and bacteriological examination, are given in Table I:—

TABLE I.

Results of test in cases of leprosy.

Inhibition of hæmolysis.	+	Per cent.	T	Per cent.	±	Per cent.	—	Per cent.	Total cases.
<i>Lepromatous type—</i>									
(Bact. Exam +)	98	83·77	8	6·84	9	7·69	2	1·7	117
<i>Neural type—</i>									
(Bact. Exam +)	7	63·63	3	27·28	1	9·09	0	0·0	11
(Bact. Exam —)	5	4·09	8	6·56	17	13·95	92	75·40	122
Total neural type	12	9·0	11	8·25	18	13·55	92	69·2	133

+ Complete, T Almost complete, ± Partial, — None.

RESULTS IN OTHER DISEASES.

In Table II we show the results of the test in five different groups of non-leprous patients, which are as follows:—

- (a) Patients showing clinical and serological evidence of syphilis (W. R. positive).
- (b) Patients with other diseases including skin diseases, showing no evidence of syphilis.
- (c) Patients with clinical evidence of tuberculosis of the lungs but with no acid-fast bacilli in the sputum.
- (d) Patients suffering from leishmaniasis, either kala-azar or dermal leishmaniasis.

TABLE II.

Results of test in other diseases.

Inhibition of hæmolysis.	+	Per cent.	T	Per cent.	±	Per cent.	—	Per cent.	Total cases.
W.R. + ..	1	2·17	2	4·34	6	13·04	37	80·45	46
Skin (W.R.) — disease.	0	..	0	..	0	..	43	100	43
Tuberculosis ..	0	..	0	..	3	100	0	..	3
Leishmaniasis ..	18	90·00	0	..	0	..	2	10·00	20

See notes under Table I.

DISCUSSION OF RESULTS IN CASES OF LEPROSY.

The first thing that strikes one in these results is the high percentage of cases of the lepromatous type showing partial or complete inhibition of hæmolysis. Of 117 cases only 2, i.e., 1·7 per cent. showed no inhibition, while 106, i.e., 90·6 per cent, showed complete or almost complete inhibition. This is in accordance with the results reported by other workers. While some inhibition of hæmolysis is recorded in practically all lepromatous-type cases, it is interesting to observe that the proportion showing complete inhibition is highest (100 per cent) in the severe cases, lowest in the slight cases, the moderate cases coming in between. This is shown in Table III.

It is interesting to observe that even cases with very slight lesions of lepromatous type usually show inhibition of hæmolysis in the test, although this is not always complete. This fact indicates how proper classification of cases of leprosy will influence the results reported in the two main types of leprosy, for in neural leprosy as is stated below the results of the test are usually negative.

TABLE III.

Results of complement-fixation test in 117 'L' cases sub-classified according to extent and severity of lesions.

Inhibition of hæmolysis.	L-1 (SLIGHT) 17 CASES.				L-2 (MODERATE) 62 CASES.				L-3 (SEVERE) 38 CASES.			
	+	T	±	-	+	T	±	-	+	T	±	-
Number of cases.	8	2	6	1	52	6	3	1	38	0	0	0
Percentage	47.0	11.8	35.3	5.9	83.4	10.2	4.8	1.6	100.0	0	0	0

See notes under Table I.

The next striking thing is the relatively low percentage of positive findings in cases of neural leprosy in which no bacilli have been found. Table I shows that of such cases only 24.6 per cent showed inhibition of hæmolysis and of these more than half showed incomplete inhibition. The results obtained, however, in cases of neural leprosy in which bacteriological examination had revealed bacilli, usually few in number, are very different. Of 11 such cases all showed inhibition of hæmolysis, and in ten of the eleven, the hæmolysis was complete or almost complete.

These findings are not in agreement with some of the reported findings of other workers, Bier and Arnold having reported 57 per cent positive, Bier 70 per cent, and Pereira 80 per cent in cases of neural type.

In general we may say that the results of the complement-fixation test in leprosy go parallel with the results of ordinary bacteriological examination of smears taken from the skin and mucous membrane. This is shown in Table IV in which the cases are classified, not according to the type of leprosy, but according to the results of bacteriological examination.

These results indicate that the W. K. K. complement-fixation test is of little value in diagnosis, for fixation of complement is seen as a rule only in cases in which clinical diagnosis presents no difficulty, and in which ordinary bacteriological examination reveals bacilli.

From the point of view of prognosis it is interesting to compare the results of the W. K. K. complement-fixation test with the results of the leprolin test. The leprolin test gives a positive result in cases of the mild neural type even when bacilli are found, and a negative result in cases of the severer lepromatous type, and hence the leprolin test is of value in classification and prognosis. The complement-fixation test, however, does not differentiate between the cases of lepromatous type with a bad prognosis, and the cases of neural type in which a few bacilli are found in smears, but in which the prognosis is usually good. Therefore, the complement-fixation test appears to be of little value even in prognosis.

TABLE IV.

Results of complement-fixation test in 250 cases of all types classified according to results of bacteriological examination.

Inhibition of hæmolysis.	MANY BACILLI FOUND 81 CASES.				MODERATE NUMBER OF BACILLI FOUND 30 CASES.				FEW BACILLI FOUND 17 CASES.				NO BACILLI FOUND 122 CASES.			
	+	T	±	-	+	T	±	-	+	T	±	-	+	T	±	-
Number of cases.	74	4	2	1	27	0	3	0	5	6	5	1	5	8	17	92
Percentage	91.4	4.9	2.5	1.2	90.0	0	10.0	0	29.4	35.3	29.4	5.9	4.1	6.6	13.9	75.4

See notes under Table I.

It might, however, be interesting to study whether, when a bacteriologically positive case becomes bacteriologically negative, the power of the serum to fix complement disappears or not. If so the test might be of value in judging whether the disease is arrested or not.

DISCUSSION OF RESULTS IN OTHER DISEASES.

The three diseases other than leprosy in which the test has given positive results are syphilis, tuberculosis, and leishmaniasis. Tests of 46 cases of syphilis with a positive Wassermann test, showed inhibition of hæmolysis in 20 per cent, but in only one case was this complete.

Only three cases of tuberculosis were tested, with partial inhibition of hæmolysis in all three. These were all cases in which no bacilli have been found in the sputum.

An interesting finding is the relatively high percentage of positive findings in cases of leishmaniasis. This is in accordance with the report of Bier who found 70 per cent of positive results in such cases. Of 17 cases of kala-azar (all aldehyde positive) all showed complete inhibition of hæmolysis. Of 3 cases of dermal leishmaniasis one showed complete inhibition.

These results indicate that the test is not specific for leprosy, that positive results can be obtained in syphilis, tuberculosis, and leishmaniasis, that a negative result may be obtained in leprosy, and in fact is usually obtained in the milder neural form of the disease.

SUMMARY AND CONCLUSIONS.

1. The literature of complement-fixation is briefly reviewed with special reference to the use of the W. K. K. antigen,

2. The results of the test in 250 cases of leprosy, 46 cases of syphilis, 20 cases of leishmaniasis, 3 cases of tuberculosis of the lungs, and 43 cases of other diseases are given.

3. Of 117 cases of leprosy of the lepromatous type, 98.3 per cent showed inhibition of hæmolysis, 83.77 per cent showing complete inhibition.

4. Of 133 cases of neural leprosy, only 41 (31 per cent) showed some inhibition of hæmolysis, only 12 of these showing complete inhibition. These 133 neural cases included 11 bacteriologically positive cases of whom all showed some fixation of complement, complete in 7 cases.

5. The results of the test in cases of leprosy are found to correspond very closely to the results of examination of the skin for *M. lepræ*. Of cases in which bacilli were found, nearly all showed some inhibition of hæmolysis in the test.

6. The test is considered to be of little value in diagnosis or prognosis, for inhibition of hæmolysis is found only in cases in which diagnosis is easy, and the test fails to differentiate between the neural cases showing bacilli in smears which often have a good prognosis, from the lepromatous cases which have a bad prognosis.

7. Of 46 cases of syphilis with a positive Wassermann test, 20 per cent showed inhibition of hæmolysis, usually incomplete. Of three cases of tuberculosis of the lungs all showed partial inhibition of hæmolysis. Of 20 cases of leishmaniasis, 18 showed complete inhibition of hæmolysis. All 43 cases of other diseases gave negative results.

8. The test is, therefore, not specific for leprosy, since in other diseases, chiefly leishmaniasis, positive results can be obtained, and since in leprosy of the mild neural type negative results are usually obtained.

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COMPLEMENT-FIXATION IN LEPROSY WITH WITEBSKY,
KLINGENSTEIN AND KUHN (W. K. K.) ANTIGEN*:
A NEW TECHNIQUE.

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[Received for publication, September 8, 1938.]

I. THE TECHNIQUE.

1. *The reagents.*

(i) *The hæmolytic system.*—This was prepared in accordance with the requirements of method No. IV of the Medical Research Committee's (1918) report on the Wassermann test with the exception that the r. b. c. suspension was standardized by a method described by Greval *et al.* (1930). The M. H. D. of the complement determined for the purposes of the Wassermann reaction, without the antigen, was also used in this technique.

(ii) *The antigen.*—A small volume of W. K. K. antigen (0.3 c.c.) was evaporated and made into a uniform suspension with two volumes of saline (0.6 c.c.). This

*The antigen was purchased from the Behring Institute, Germany. It is essentially a solution of human tubercle bacillus in benzol, after extraction with alcohol, pyridin, and acetone.

was the starting point as recommended by the makers of the antigen. From this suspension were prepared dilutions of 1 in 10, 1 in 15, and 1 in 20, and tested for anti-complementary activity with 1 M. H. D. of complement, thus:—

Tube.	1.	2.	3.
Antigen dilution, 1 vol. of:—	1 in 10	1 in 15	1 in 20
Complement dilution containing 1 M.H.D., in a volume:—	1 vol.	1 vol.	1 vol.
Saline	1 vol.	1 vol.	1 vol.
	Left at room temperature $\frac{1}{2}$ hour and at 37°C. $\frac{1}{2}$ hour.		
Suspension of sensitized r. b. c.	1 vol.	1 vol.	1 vol.
	Left at 37°C. for $\frac{1}{2}$ hour.		

The volume employed was 0.25 c.c.

The strongest dilution of the antigen permitting a complete lysis of r. b. c. was taken as the dose for the test. The strength varied, from 1 in 10 to 1 in 20, with the samples of the preparation used. The titration can be prolonged in the same arithmetical series (1 in 25, 1 in 30,.....) if necessary.

The titration for the anti-complementary activity of the antigen dilution was not repeated in full every time the test was put up. A tube containing one dose of the antigen dilution was put up with 1 M. H. D. of the complement and included in the rack as the antigen control.

2. *The serum for the test.*

This was inactivated at 55°C. for 30 minutes and diluted 1 in 5, as is done in Wassermann reaction according to the aforesaid method of the Medical Research Committee (now Council).

3. The test.

(i) An ensemble:—

Tube.		1. Serum control.	2. Test proper.
Serum dilution	..	1 vol.	1 vol.
Antigen dilution	..	Nil	1 vol.
Saline	1 vol.	Nil.
Complement dilution, containing 2 M. H. D. in a volume.		1 vol. "	1 vol.
		Left at room temperature $\frac{1}{2}$ hour and at 37°C. $\frac{1}{2}$ hour.	
Suspension of sensitized r. b. c.		1 vol.	1 vol.
		Left at 37°C. for $\frac{1}{2}$ hour.	

The volume employed was 0.25 c.c.

(ii) *Reading of results.*—The antigen control and the serum control being free from inhibition of lysis, the inhibition in the other tube was read as follows:—

Complete inhibition of lysis	= +	} positive.
A trace of lysis	= T	
More than a trace of lysis	= ±	
Lysis almost complete, only turbidity present, no coloured deposit, no difference in colour between this tube and the next	= ? -	} negative.
Lysis complete	= -	

II. REMARKS ON THE TECHNIQUE.

I. Special features.

In devising this technique we have taken into consideration (i) availability of an antigen of a constant potency, (ii) superiority of methods of complement-fixation

TABLE II.

25 controls.

Classification according to diseases.	Number of cases.	Results according to Witebsky, Klingenstein and Kuhn's technique.	Results according to our technique.
FROM THE SKIN DEPARTMENT, SCHOOL OF TROPICAL MEDICINE.			
Seborrhœa	2	—	—
Leukoderma	2	—	—
Psoriasis	2	—	—
Tinea cruris	1	—	—
Pustular folliculitis	1	—	—
Syphilis	1	—	—
Lupus vulgaris	3	—	—
FROM THE IMPERIAL SEROLOGIST'S LABORATORY.			
Strongly positive W. R.	9	—	—
Strongly positive W. R.	1	±	±
Negative W. R.	3	—	—
TOTAL ..	25

The results are identical.

References to the previous work on the subject by other workers; tables summarizing 250 cases tested by our method so far; and additional remarks on the sensitiveness, specificity, and utility of the reaction in relation to the clinical and bacteriological findings of the cases, will be found elsewhere in this issue of the *Journal* (Lowe and Greval, 1939).

SUMMARY.

1. A technique of complement-fixation for leprosy has been devised using the hæmolytic system of the method No. IV of the report of the (British) Medical Research Committee (now Council) on the Wassermann test, and W. K. K. antigen.

2. The sensitiveness of the reaction in lepromatous type of the disease is of a very high order. Like a strongly positive Wassermann reaction of secondary syphilis, a positive reaction is not likely to be missed. The sensitiveness in neural leprosy is low.

3. The specificity of the reaction is not of a very high order. Kala-azar gives a positive reaction. Doubtful reactions have sometimes been obtained in malaria, syphilis, dermal leishmaniasis, and tuberculosis. A W. R. positive case has also given a + reaction.

4. The utility of the reaction is considerable in the diagnosis of the lepromatous type of the disease, but little in the diagnosis of the neural type. It may help in evaluating arrest of the disease in previously positive cases.

5. The procedure involved in putting up the test is brief and the reagents used standardized. A quantitative reading can be easily obtained.

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Postscript.—

As a result of further work with the W. K. K. antigen, after this communication was sent for publication, it has been found that the reaction in kala-azar is even stronger than the reaction in the lepromatous type of leprosy. One in 200 dilutions of many sera have given a + reaction. Four sera from suspected cases giving a doubtful or negative reaction with formaldehyde have given a strong reaction with the antigen. These cases, later, have been proved to be positive by sternum, liver, or spleen puncture.

A + reaction has also been obtained in cases of tuberculosis but mostly in a dilution of 1 in 5 of the sera. Out of 25 sera only 1 gave the reaction in 1 in 50, 1 in 1 in 12.5, and 7 in 1 in 5. Eight gave a ± reaction in 1 in 5. Eight were negative.—S. D. S. G. *et al.* (7th December, 1938).

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DIFFERENTIAL ISOLATION OF *V. CHOLERÆ*.

BY

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[Received for publication, December 31, 1938.]

EXPERIENCE in the isolation of vibrios from water and other sources during the past three years by the use of alkaline peptone-water enrichment followed by plating on Aronson's medium has shown that, while the solid medium suppresses the growth of non-vibrio types with the exception of certain streptococci, the enrichment medium allows the growth of many types prejudicial to the growth of the vibrio. The degree to which this takes place depends on the extent to which the initial alkalinity is maintained in the process of growth.

A further disadvantage when these methods are employed for the isolation of *V. cholerae* is that, even where the reaction is maintained sufficiently alkaline to reduce the growth of *coliform* and other organisms, all vibrio types are facilitated and the presence of certain inagglutinable vibrio types in strong peptone solution may rapidly reduce the number of *V. cholerae* in an inoculum to a point at which isolation is difficult or impossible. A description is given in this paper of methods that have been employed in an attempt to deal with these difficulties.

MAINTENANCE OF REACTION OF THE ENRICHMENT MEDIUM.

Use of stoppered bottles.—It has been found that in 1 per cent peptone and 1 per cent NaCl the range of hydrogen-ion concentration at which maximum multiplication of *V. cholerae* in pure culture can occur is from 6·0 to 9·0 and that good multiplication can be secured at a pH in excess of 9·4. It was decided that a pH of 9·2 was most suitable for the differentiation of *V. cholerae* from *coliform* organisms. This pH cannot be maintained in open flasks and tubes owing to absorption of carbon dioxide from the air. Even when a medium of this pH is inoculated with *V. cholerae* alone in stoppered bottles the pH drops to 8·2 to 8·6 within 24 hours. When a carbohydrate is added to the peptone the reduction of pH is of course accentuated. The use of stoppered bottles, however, eliminates the spontaneous reduction of pH which the medium undergoes in tubes or flasks and may, therefore, be considered a useful procedure.

Buffer solutions.—Neither the addition of alkaline sodium phosphate nor glycocoll-NaOH mixtures is effective in a medium containing carbohydrate in maintaining a pH in excess of 6.8 after 24 hours inoculation of a heavy inoculum especially when *coliform* organisms are present.

Supplementary addition of alkali.—A most useful procedure, especially when attempting to isolate from small inocula, is the addition at intervals of small quantities of alkali to restore the pH. *Vibrio* multiplication decreases directly with the increased growth of the *coliform* types that occur with lowering of pH. In the early stages of incubation, therefore, they will multiply relatively rapidly and if the alkaline reaction is restored after, say, 3 to 5 hours incubation, the surviving *coliform* organisms will have a larger number of *vibrio* types to compete with than existed at the moment of inoculation.

VALUE OF FERMENTABLE CARBOHYDRATE IN DIFFERENTIATION BETWEEN *VIBRIO* TYPES.

The work of Stephens and Whetham (1924) showed that the growth of *Bact. coli* during the first hours of multiplication in a glucose-containing synthetic medium was largely dependent on utilization of glucose. Dr. A. N. Goyle of this Institute in an unpublished experiment showed that the agglutinable *vibrio* could be isolated in almost pure form from an inoculum containing that *vibrio* and a non-mannose-fermenting *vibrio*, when mannose was added to simple peptone water and other media.

It was decided, therefore, to add mannose to the enrichment medium, as it has been shown that by using normal media half or more of the *vibrios* naturally found in water fail to ferment this sugar.

EXPERIMENTAL RESULTS.

The results of the use of media for differential isolation in which the principles employed were: (a) the maintenance of alkalinity for the suppression of the growth of *coliform* organisms and (b) the use of mannose for facilitating the growth of *vibrios* fermenting this sugar, are shown in the tables. In Table I a repetition of Goyle's experiment done in buffered peptone water containing mannose shows that the agglutinable *vibrio* can be isolated from a combined inoculum of that *vibrio* and mannose-non-fermenting *vibrios* in a proportion of 1/10,000 as a pure or almost pure culture after 24 hours incubation. On the other hand, as shown in Table II, when the inoculum along with the agglutinable *vibrios* was an inagglutinable mannose-fermenting *vibrio*, the latter almost completely outgrew the former. Tables III and IV show the results of the use of peptone water containing mannose and buffered by sodium phosphate or glycocoll with varying doses of an agglutinable *vibrio*, a mannose-non-fermenting *vibrio* and *coliform* organisms. With a moderate inoculum of agglutinable *vibrio* and heavy opposing inocula of the other organisms it was not possible to isolate with certainty from inocula containing *Bact. coli* and in no case from inocula containing *Bact. aerogenes*. With a lower inoculum of agglutinable *vibrio* but with a smaller opposing inoculum isolation could be effected, though not with certainty in the case of *Bact. aerogenes*. Table V shows the results

of similar experiments repeated using a fresh stool inoculum in place of *coliform* organisms together with repeated re-adjustment of the reaction. Isolation could not be effected under severe conditions.

It was obvious from these results that under conditions likely to exist in the field the use of these methods could not be expected to yield satisfactory isolation from natural waters and stools of carriers, where small concentrations of *V. cholerae* together with heavy opposing inocula might be expected.

A new line of approach was developed, the basis of the study being the bismuth-sulphite enrichment medium of Wilson and Blair (1931), which was modified in the first instance by the omission of brilliant green, increase of pH to 9.2, substitution of mannose for mannite in 1 per cent concentration, and replacement of broth by peptone water.

The results with this medium have been included for comparison with those of buffered peptone in Tables III, IV, and V and it will be seen that greatly improved results are obtained with inoculum containing *Bact. aerogenes* and stool suspensions. The maintenance of reaction was also remarkable with the medium.

It soon, however, became evident that, while isolation from mixtures containing the agglutinable vibrio and other organisms, when *V. cholerae* alone or with a very small opposing inoculum was employed, the vibrio could only be isolated with difficulty and never from a small inoculum in the region of 14 organisms per c.c. of the medium employed. If the pH was relaxed to 7.6, then isolation was possible, but this destroyed the full differential character of the medium. Table VI shows that this method was satisfactory using a small inoculum and without any addition of alkali subsequent to inoculation against from 2.5 c.c. to 0.05 c.c. of stool. When the stool inoculum was 0.005 c.c. or lower no isolation was effected, while autoclaving the stool also prevented isolation.

As this was obviously a very undesirable state of affairs attempts were made to modify the medium with a view to removing this inhibitory effect on the growth of *V. cholerae*.

Table VII shows the effect of doubling the concentration of liq. bismuthi. It was found that isolation could be effected from 5.0 c.c. of stool with a small inoculum but the inhibitory effect in the presence of small stool inocula remained.

Table VIII shows the effect of liq. bismuthi of triple strength. In this case, in seven experiments in which the vibrio was inoculated in pure culture, the vibrio was recovered on three occasions only, but the plates even then showed that a marked inhibition of growth was still present.

Table IX shows the effect of still further increasing the strength of the liq. bismuthi.

Further attempts at modification consisted in the omission of alcohol, mercury perchloride, reduction of the concentration of sodium sulphite and mannose. It was found that reduction of the sodium sulphite to 1/10th, omission of the alcohol, and reduction of the mannose to 0.5 per cent all assisted in the chances of the vibrio surviving in the absence of stool. The isolation from faeces, however, was less satisfactory and details of these experiments are not given.

The next attempt was to substitute for the sodium chloride of the original formula a combined mixture of salts in the following proportion: NaCl 27 parts, KCl 1 part, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ 3 parts, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1.75 parts, which is roughly the proportion in which these salts occur in sea-water. A total concentration of salts of 2 per cent was adopted. With this mixture isolation was effected from as low as 0.14 organisms per c.c. of media inoculated against 5 c.c. of stool, while in the presence of 4,750 non-mannose-fermenting vibrios per c.c. of medium isolation was effected from 1.4 agglutinable vibrio per c.c. of medium and 5 c.c. of stool; and in the presence of 48 mannose-fermenting vibrios per c.c. of medium containing 5 c.c. of stool from 14 agglutinable vibrios per c.c. of medium. The maintenance of alkalinity was also better than in any other mixture.*

A few experiments are given in Table XI showing results from artificially infected natural waters. The first four experiments were done in mannose-phosphate-peptone with no attempt at concentrating the organisms in the water. With the exception of the Kasauli spring water these waters contained inagglutinable vibrios and a large *coliform* count. The results were disappointing. Using the bismuth-sulphite original modification and passing one litre of water through a Seitz filter and inoculating the disc better results were obtained, especially when 2 per cent NaCl were added to the water and the reaction raised to 9.2 immediately after inoculation. Under the supposition that the vibrio might be dying off from lack of nutritive pabulum in the water (the average Tidy figure of the water in the Kasauli and surrounding districts being much lower than that in the endemic areas of Bengal, the examination of whose waters this study was designed to facilitate) experiments were instituted by inoculating an artificial water consisting of 1/50,000 peptone solution and 1 per cent sea-salt mixture, which has a Tidy figure rather below the average of Bengal waters. The technique was also simplified by the use of concentration on filter-papers impregnated with Kieselguhr. From 1 to 5 hours were required to pass 3 to 15 litres through 6-inch funnels fitted with the impregnated filter-paper during which time it may be supposed that some multiplication was taking place in the inoculated solution but at the low temperature existing in Kasauli during the winter months (about 50°F. on the bench) this might be considered negligible. The exact technique is given later. The results of this method are shown in Table XII. It appears that under these conditions very little loss of organisms occurs and vibrios can be recovered from a very high dilution.

CONCLUSION.

The results given above show that the final formula for a differential isolation medium adopted is likely to be adequate to all needs with the important exception that the growth of mannose-fermenting vibrios of all types will be facilitated and it is not, therefore, certain that *V. cholerae*, if present, will not be outgrown by the inagglutinable vibrios present which ferment this sugar. Moreover, a preliminary field trial of the original modification of the bismuth-sulphite medium showed that

*Later work has shown that NaCl in a concentration of 2 per cent can be substituted for the above mixture of salts without greatly interfering with the results. At the same time other experimental work has shown the enhanced value of the salt mixture under slightly different conditions.

using this method the percentage of Calcutta water samples from which mannose-fermenting inagglutinable vibrios were isolated was raised to 72 per cent. The full value of the method and the importance of this defect can only be elucidated by an extension of a field trial, which has already shown promising results.

Clearly what is required is a carbohydrate specifically fermented by *V. cholerae*.

TECHNIQUE OF DIFFERENTIAL ISOLATION.

The following is the method finally selected for differential isolation :—

Bismuth-sulphite media.—Fifty c.c. screw-capped medical bottles were employed except for the inoculation of Seitz discs and filter-paper when 100 c.c. wide-mouthed glass-stoppered flasks were substituted. All ingredients were made up in separate solutions and kept in stoppered bottles. Two per cent peptone solution and distilled water were the only solutions that were sterilized. Salt mixtures remain sterile without sterilization, at any rate sufficiently so for purposes of this media. Mannose was made up in 10 per cent solution for the needs of the day and boiled. Sodium sulphite was made in 20 per cent solution and slightly warmed to dissolve only. The media are thus very easily prepared and form fresh solutions.

The formula of the final modification was :—

	c.c.
Peptone, 2 per cent	8.8
Sea-salt mixture	1.2
Aq. dest. or stool emulsion	10.0
Mannose, 10 per cent	1.0
Liq. bismuthi	0.12
Sodium sulphite, 2 per cent	1.2
Absolute alcohol	0.2
Mercury perchloride, 1/10,000	0.8

The formula of the sea-salt mixture was :—

	Parts.
NaCl	27
KCl	1
MgCl ₂ ·6H ₂ O	3
MgSO ₄ ·7H ₂ O	1.75
Aq. dest.	100

Liq. bismuthi was prepared as indicated by Wilson and Blair (*loc. cit.*).

The stool emulsion was prepared by measuring out the required volume of stool in the barrel of a Roux syringe and mixing with an equal quantity of normal saline and filtering through a single layer of lint. This mixture could then be diluted as required so that the volume added to the media bottle was 10 c.c.

Aronson's medium was made up to the standard formula except that 5/6th the normal quantity of Na_2CO_3 was added. The alkali was weighed out on a chemical balance to the nearest milligram. Plates were stored in complete darkness in the cold room.

Agar plates were also employed in each experiment as it was found that where very few vibrio colonies were present, they could be picked off with greater ease from the agar plates than from the Aronson's medium. With the formula finally adjusted the differential action of Aronson's medium was not really required, but the latter medium is very useful in the field as it maintains sterility as long as the medium is fit for use.

ADJUSTMENT OF REACTION.

To test-tubes 3 inches \times 0.3 inch containing 1.5 c.c. of aq. dest. and 0.2 c.c. of 0.04 per cent thymol-blue solution was added 0.1 c.c. of the medium. N/1 NaOH was then added to the medium until on the transfer of 0.1 c.c. to the reaction tube prepared as above the first true blue colour appeared. This gives a pH somewhere in excess of 9.2. A useful check for inexperienced workers was found to be by adding one drop of alkali to the reaction tube after the addition of the media. If the colour reached by the medium is well short of the full blue colour of the indicator produced by the additional drop of alkali, then the reaction is correct. Bottles were then incubated at 37°C. and in most cases brought back to the initial reaction after 3 to 5 hours. The latter procedure was of considerable value and should not be omitted. It is extremely difficult or impossible to work with this indicator by ordinary artificial light.

INOCULUM.

A standard vibrio was used throughout TMCH 1800/1 isolated in Calcutta in 1937 from a typical case of cholera. The vibrio conformed in all respects to the Inaba type, group I of Gardner and Venkatraman (1935). Occasional experiments were done with recently isolated vibrios, which as a rule show greater growth activity than laboratory maintained cultures. The mannose-fermenting inagglutinable vibrio usually employed for comparative test was W9E, a Punjab water strain, Heiberg type II sugar reactions, cholera-red and Indol reactions positive, and an active grower.

The *Bact. coli* and *Bact. aerogenes* cultures were old stock cultures from the stock of the Institute, the latter being an especially active grower.

Twenty-four hours broth cultures or agar washings standardized to No. 3 Brown's opacity tube diluted in normal saline were used as inocula.

CONCENTRATION OF ORGANISMS FROM WATER.

Where Seitz filters were employed water was run through by gravity through the pressure type filters using about six feet head of pressure.

Where Kieselguhr filtration was employed the method was similar to that used by Asheshov (1933) except that ordinary open-glass funnels were employed there being no necessity to maintain sterility. Three hundred c.c. of 0.5 per cent Kieselguhr were run through a 6-inch filter-paper. The water was held in large 4-litre flasks closed with a rubber cork containing a moderate-sized glass tube. These were inverted in the funnel in such a manner that the outer end of the tube just reached below the surface of the fluid in the funnel. Filtration then proceeded automatically until the flask was emptied. About 15 litres could be passed through two filter-papers in 5 to 6 hours. The filter-papers were then folded and placed in 60 c.c. or so of the bismuth-sulphite enrichment medium and incubated overnight.

SUMMARY.

1. Various methods are described and investigated with a view to the differential isolation of *V. cholerae* from mixtures of cultures, stools, and natural and artificial waters inoculated with *V. cholerae*.

2. A modification of the bismuth-sulphite enrichment medium of Wilson and Blair (*loc. cit.*) was the best and enabled the vibrio to be isolated from an inoculum that would only just grow in ordinary broth.

3. By the use of this medium mannose-fermenting vibrios can be successfully differentiated from non-mannose-fermenting vibrios and from *coliform* types. Other common water and stool organisms except streptococci are suppressed but no method of facilitating *V. cholerae* as against mannose-fermenting inagglutinable vibrios was discovered. The value of the method will depend on whether the mannose-fermenting vibrios found in natural sources can outgrow *V. cholerae* or not. The difficulty due to the growth of total organisms when ordinary peptone-water enrichment is employed is overcome.

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TABLE I.

Growth of mannose-fermenting and mannose-non-fermenting vibrios in Mannose-Phosphate-Peptone.

Experiment number.	Medium.	Initial pH.	Mannose-fermenting vibrios.	Mannose-non-fermenting vibrios.	Isolation at 5 hours.	Isolation at 24 hours.
87	MPP	9.0	Ogawa	R a n g o o n 'rough'.	R a n g o o n 'rough', 100 per cent.	Ogawa, vast majority.
87	"	9.0	Lahore 1	TMCH 630/3 T	TMCH 630/3 T, 100 per cent.	Lahore 1, 100 per cent.
87	"	9.0	W 880	1612	1612, 100 per cent.	W 880, 100 per cent.
87	"	9.0	TMCH 319 GI.	TMCH 324/7	TMCH 324/7, 100 per cent.	TMCH 319 GI, 99 per cent.
87	"	9.0	Inaba	584 Karachi	584 Karachi, 100 per cent.	Inaba, 99 per cent.

For description of above organisms see Taylor, Read and Pandit (1937).

TMCH 319 GI was a recently isolated agglutinable vibrio from a Calcutta case.

584 Karachi was a vibrio isolated from a case of cholera in Karachi in 1931, showing Heiberg type II sugar reactions.

MPP = Mannose-Phosphate-Peptone water (mannose 1 per cent, Na_2HPO_4 1 per cent, NaCl 0.5 per cent, peptone 1 per cent in aq. dest., pH 9.0, 24 c.c. in screw-capped bottles).

N.B.—In each case the inoculum of mannose-non-fermenting organisms was 10,000 as strong as that of the mannose-fermenting organisms.

TABLE II.

Growth of mannose-fermenting vibrios of different types in Mannose-Phosphate-Peptone.

Experiment number.	Medium.	Initial pH.	Inoculum of inagglutinable mannose-fermenting vibrios.	Inoculum of agglutinable vibrios.	Isolations at 24 hours.
116	MPP	9.2	CTM 5780	TMCH 1800/1	CTM 5780, majority TMCH 1800/1, a few.
123	"	9.2	Lahore 1	"	L a h o r e 1, 100 per cent.

Abbreviations and notes as in Table I.

N.B.—In each case the inoculum of both organisms was approximately equal.

TABLE III.

Isolations from mixtures containing Bact. coli (typical experiments only quoted).

Medium.	Experiment number.	Reaction of medium adjusted up to hours.	<i>V. cholerae</i> inoculum per c.c. of media. Number of vital organisms.	Mannose-non-fermenting inagglutinable vibrios per c.c. of media. Number of vital organisms.	<i>Bact. coli</i> per c.c. of media. Number of vital organisms.	Number of <i>V. cholerae</i> isolated.
Mannose, 1 per cent Na ₂ HPO ₄ , 1 per cent NaCl, 5 per cent Peptone, 1 per cent pH 9.2 24 c.c. in screw-capped bottles (MPP)	105	0	1,400	56,000,000	102,000,000	A few.
	88	1	1,400	56,000,000	102,000,000	Nil.
	95	4	1,400	56,000,000	102,000,000	Several.
	91	3	1,400	56,000,000	102,000,000	Nil.
	98	6	30	1,100	20,000	99 per cent.
	120	0	30	1,100	20,000	Majority.
	94	8	14	56,000,000	102,000,000	Nil.
Mannose, 1 per cent Glycocoll, 2 per cent NaCl, 0.5 per cent Peptone, 1 per cent pH 9.2 26 c.c. in screw-capped bottles (MPG)	106A	0	1,400	56,000,000	102,000,000	Many.
	133	0	30	1,100	20,000	99 per cent.
	107A	0	3	1,100	20,000	99 per cent.
	108A	0	0.03	1,100	20,000	Nil.
Modified bismuth-sulphite medium Peptone, 1 per cent NaCl, 0.5 per cent Mannose, 1 per cent Liq. bismuthi, 0.04 c.c. Sodium sulphite, 20 per cent, 1.2 c.c. Absolute alcohol, 0.2 c.c. HgCl ₂ , 1/10,000, 0.8 c.c. pH 9.2 24.2 c.c. in screw-capped bottles (W and B modified)	145	0	1,400	56,000,000	102,000,000	100 per cent.

TABLE IV.

Isolations from mixtures containing Bact. aerogenes (typical experiments only quoted).

Medium.	Experiment number.	Reaction of media adjusted up to hours.	<i>V. cholerae</i> inoculum per c.c. of media. Number of vital organisms.	Mannose-non-fermenting inagglutinable vibrios per c.c. of media. Number of vital organisms.	<i>Bact. aerogenes</i> per c.c. of media. Number of vital organisms.	Number of <i>V. cholerae</i> isolated.
MPP .. {	96	8	1,400	56,000,000	102,000,000	Nil.
	102	0	30	1,100	20,000	A few.
MPG .. {	133	8	1,400	56,000,000	102,000,000	Nil.
	134	0	30	1,100	20,000	Nil.
W and B modified.	150	0	1,400	56,000,000	102,000,000	Many.

Abbreviations as in Table III.

TABLE V.

Isolations from mixtures containing faeces (typical experiments only).

Medium.	Experiment number.	Reaction of media adjusted up to hours.	<i>V. cholerae</i> inoculum per c.c. of media. Number of vital organisms.	Mannose-non-fermenting inagglutinable vibrios per c.c. of media. Number of vital organisms.	c.c. stool.	Number of <i>V. cholerae</i> isolated.
MPP 24 c.c. .. {	153	8.5	1,400	56,000,000	3.2	Nil.
	114	21	30	1,100	3.2	Nil.
	164B	8.75	30	1,100	3.2	A few.
	100	60	30	1,100	1.6	Several.
	100	60	30	1,100	0.016	Majority.
MPG 26 c.c. ..	146A	33	30	1,100	3.2	Nil.

Abbreviations as in Table III.

TABLE V—concl'd.

Medium.	Experiment number.	Reaction of media adjusted up to hours.	<i>V. cholera</i> inoculum per c.c. of media. Number of vital organisms.	Mannose-non-fermenting inagglutinable vibrios per c.c. of media. Number of vital organisms.	c.c. stool.	Number of <i>V. cholerae</i> isolated.
W and B modified 2+2 c.c.	151	4	1,100	56,000,000	3·2	Many.
	152	4	30	1,100	3·2	100 per cent.
	159A*	24	30	Nil	3·2	100 per cent.
	156A	0	14	5,600	3·2	Many.
	175†	72	14	Nil	3·2	2 colonies.
	157	0	1·4	5,600	3·2	A few.

* Stool and inoculum kept for 10 days in 2 per cent NaCl.

† Stool and inoculum kept as above for 6 days.

Abbreviations as in Table III.

TABLE VI.

Isolations from small inocula of V. cholerae with varying doses of stool.

Medium.	Experiment number.	Reaction of media adjusted up to hours.	<i>V. cholerae</i> inoculum per c.c. of media.	c.c. stool.	<i>V. cholerae</i> isolated.
W and B modified	320	2	14	5·0	Nil.
	320	2	14	2·5	Nil.
	322	0	14	2·5	Many.
	324	0	14	0·05	Many.
	324	0	14	0·005	Nil.
	339	0	14	1·25	..
	342	0	14	autoclaved Nil.	Nil.*

* Media at 7·6 pH was positive.

Abbreviations as in Table III.

TABLE VII.

Isolations using double strength liq. bismuthi.

Medium.	Experiment number.	Reaction of media adjusted up to hours.	<i>V. cholerae</i> inoculum per c.c. of media.	c.c. stool.	<i>V. cholerae</i> isolated.
W and B modified (Liq. bismuthi, 0.08 c.c.)	323	0	14	5.0	1 colony.
	333	0	14	0.0005	A few in pure culture.
	337	0	14	0.0005	Nil.
	337	0	14	0.00005	Nil.
	342	0	14	Nil	Nil.

Abbreviations as in Table III.

TABLE VIII.

Isolations using triple strength liq. bismuthi.

Medium.	Experiment number.	Reaction of media adjusted up to hours.	<i>V. cholerae</i> inoculum per c.c. of media.	c.c. stool.	<i>V. cholerae</i> isolated.
W and B modified (Liq. bismuthi, 0.12 c.c.)	340	0	14	5.0	Many.
	354	0	14	5.0	Nil.
	358	0	14	2.5	Nil.
	359	0	14	1.25	Many.
	340	0	14	0.00005	A few in pure culture.
	340	0	14	Nil	A few in pure culture.
	360	0	14	Nil	Nil.

Abbreviations as in Table III.

TABLE IX.

Medium.	Experiment number.	Reaction of media adjusted up to hours.	<i>V. cholerae</i> inoculum per c.c. of media.	c.c. stool.	<i>V. cholerae</i> isolated.
W and B modified (Liq. bismuthi, 0.16 c.c.)	360	0	14	1.25	A few.
(Liq. bismuthi, 0.16 c.c.)	360	0	14	Nil	Nil.
(Liq. bismuthi, 0.20 c.c.)	360	0	14	1.25	A few.
(Liq. bismuthi, 0.20 c.c.)	360	0	14	Nil	Nil.
(Liq. bismuthi, 0.40 c.c.)	363	0	14	1.25	Several.
(Liq. bismuthi, 0.40 c.c.)	363	0	14	Nil	A few.

Abbreviations as in Table III.

TABLE X.

Isolations from bismuth-sulphite medium containing sea-salts.

Medium.	Experiment number.	Alkalized to hours.	Agglutinable vibrio inoculum per c.c. of media.	Mannose-fermenting in-agglutinable vibrios per c.c. of media.	Non-mannose-fermenting inagglutinable vibrios per c.c. of media.	c.c. stool.	Stool grew to dilution of from 0.2 c.c. in McKonkey's broth.	Agglutinable vibrio isolation.
Peptone, 2 per cent, 8.8 c.c. Sea-salts, 2 per cent, 1.2 c.c. Stool emulsion, 10 c.c. Liq. bismuthi, 0.12 c.c. Sodium sulphite, 20 per cent, 1.2 c.c. Mannose, 10 per cent, 1 c.c. Absolute alcohol, 0.2 c.c. HgCl ₂ , 1/10,000, 0.8 c.c. pH 9.2 23.3 c.c. in screw-capped bottles.	371	3	14	1.25	10 ⁻⁵	99 per cent.
	372	3	14	1.25	10 ⁻⁵	Several.
	373	3	14	2.5	10 ⁻⁵	Majority.
	373	3	14	5.0	10 ⁻⁵	Many.
	375	3	14	5.0	10 ⁻⁴	100 per cent.
	380	6	14	5.0	10 ⁻³	99 per cent.
	380	6	14	..	475	5.0	10 ⁻³	Majority.
	380	6	14	48	..	5.0	10 ⁻³	A few.
	381	4	14	48	..	5.0	10 ⁻⁶	Several.
	380	6	14	Nil	..	Good growth.
	371	3	14	Nil	..	Good growth.
	372	3	14	Nil	..	Good growth.
	375	5	14	Nil	..	Good growth.
	375	5	1.4	5.0	10 ⁻⁴	100 per cent.
	376	3	1.4	5.0	10 ⁻⁶	Vast majority.
	381	4	1.4	..	4,750	5.0	10 ⁻⁶	Many.
	375	5	1.4	Nil	..	Good growth.
	376	3	0.14	5.0	10 ⁻⁶	Many.
	376	3	0.14	Nil	..	A few.
	381	4	0.014*	5.0	10 ⁻⁶	Nil.
	381	4	0.014*	Nil	..	Nil.

* Inoculum failed to grow in ordinary broth.

THE *COLI AEROGENES* INDEX OF POLLUTION USED IN THE BACTERIOLOGICAL ANALYSIS OF WATER.

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THE routine examination of water for indices of pollution usually comprises (1) a total count of all organisms growing on agar at 37°C., (2) the enumeration and differentiation of the *coliform* bacilli present after preliminary enrichment in MacConkey's broth followed by biochemical tests on a few of the organisms isolated from sub-cultures on solid media, and (3) a test for the presence of vibrios in 100 c.c. of the water.

The work of the past 30 years has tended to show that amongst the organisms producing acid and gas in MacConkey's bile-salt-lactose-neutral-red broth, a distinction can be made between the typical faecal *B. coli* and the *B. aerogenes*. The latter being found predominantly in cultivated soil and on vegetation is usually considered to be non-faecal in origin. Further, there are a number of intermediate types, and even *B. aerogenes* has often been found in the intestinal canal (having probably gained access thereto through the food) in comparatively small numbers. It becomes, therefore, difficult to assess the real significance of the presence of this bacterium and of the intermediate types, as indicative of excremental pollution. A differentiation between *coli* and *aerogenes* would still appear to be very desirable, if only to correlate the finding with the other data obtained from a simultaneous chemical analysis and from a topographical examination of the source. Clemesha (1908, 1912) who was one of the earliest workers to investigate the viability of the *coli-aerogenes* group found that while *B. coli* was rapidly destroyed on exposure to Indian sunlight, *B. aerogenes* and *B. cloacæ* had greater powers of resistance, especially the latter. Thresh *et al.* (1933) agree with Clemesha as, in their experience, *B. coli* which predominated at first usually tended to disappear rapidly, giving

place to *B. aerogenes* which became numerically superior and more persistent. Levine (1921) who summarized the findings of many workers showed that *B. coli* represented 93·4 per cent and *B. aerogenes* only 6·6 per cent of the *coliform* organisms in faeces, while in soil, grains, etc., the proportion of *coli* to *aerogenes* was as 13·5 : 86·5 per cent.

Cunningham and Raghavachari (1924, 1926) found that *B. coli* (M. R. +, V. P. —) constituted 97·9 per cent and *B. aerogenes* (M. R. —, V. P. +) 2·1 per cent of the *coliform* bacteria in human faeces, that the percentage ratio of *coli* : *aerogenes* was 99·5 : 0·5 in bovine faeces, 16·4 : 83·6 in soil, and 81·6 : 18·4 in milk. In natural waters of the Madras Presidency, this ratio was found to be 70·2 : 29·8 (being the same for both unfiltered and filtered waters). These authors concluded that no clue as to the probable source of organisms found in water, soil, and milk was obtainable from the grouping based on the M. R. and V. P. tests.

Raghavachari (1926) showed that the application of Koser's citrate-utilization test, along with the M. R. and V. P. tests, to sanitary water analysis in the tropics was beset with peculiar difficulties, in that many waters showing lactose fermenters in 60 c.c. and containing *B. vesiculosus* (M. R. +, indole +, V. P. —, citrate —) would have to be condemned as showing faecal pollution while some waters showing the presence of lactose fermenters in 0·01 c.c. and containing only *B. lactis aerogenes* (M. R. —, indole —, V. P. +, citrate +) might be certified as fit for use.

A number of investigators in different countries studied Eijkman's method for differentiating between faecal and non-faecal *coli*. Eijkman (1904*a, b*; 1914) introduced a glucose medium in which *B. coli* but not *B. aerogenes* was said to be able to produce acid and gas when incubated at 46°C. There has probably been no test used in the differentiation of the *coliform* organisms on which opinion has been so divided as on the Eijkman test. Some workers have considered this a sensitive and satisfactory test, while others have condemned it in unequivocal terms. A few others have suggested modifications either in the composition of the medium or in the temperature of incubation or in both which would make the test useful and specific for the purpose. Thus, Leiter (1929), Perry (1929), Williams, Weaver and Scherago (1933), Levine, Epstein and Vaughn (1934), Skinner and Brown (1934), and Minkevich *et al.* (1936*a, b*) have found either the original test or one of its modifications to be quite suitable for differentiating between faecal and non-faecal *coli*. On the other hand, Rucchoft *et al.* (1931), Burke-Gaffney (1932), Webster and Raghavachari (1934), and Webster (1935) have reported unfavourably on its usefulness.

Wilson *et al.* (1935), in the course of their critical study on the 'Bacteriological grading of milk', used MacConkey's broth and incubated their sub-cultures in a water-bath (instead of in an incubator) and maintained the temperature constantly and accurately at 44°C. Under these conditions, they found the test to be of very considerable value, and superior to all other tests in picking out a high proportion of faecal *coli* strains with rapidity. Consequently, they described four methods for the quantitative estimation of *coli-aerogenes* organisms in milk, water, or other liquid, and after discussing the objections and limitations to the ordinary dilution and the direct-plate-count methods they finally advocated the use of the dilution method with certain modifications, for use in water analysis and for research purposes.

Wilson *et al.* (*loc. cit.*) have stated that the presumptive *coliform* count is estimated from the number of positive tubes of MacConkey's broth at 37°C., the faecal *coli* count from the number of positive tubes of MacConkey's broth at 44°C. and the intermediate-*aerogenes-cloacæ* count from the number of positive tubes of citrate at 37°C. and that this method is to be recommended if all three counts are desired and is of particular value in determining the *coli-aerogenes* ratio. They have also found it convenient to express the results in terms of the probable number of organisms per unit quantity of the material under test. 'There is at present no really accurate method for ascertaining the numbers of *coliform* organisms in water. Even with the technically satisfactory modification of the dilution method advocated by them they admit that the results must always be regarded as merely approximate, in view of the existence of such an inherently large sampling error' (Wilson *et al.*, *loc. cit.*).

Bardsley (1937), however, concluded a paper read before a meeting of the Pathological Society on the 'Numerical estimation of *coliform* bacilli in water' by saying that 'the above method is much more delicate for intermediate-*aerogenes-cloacæ* and to some extent for *coli* also, that it enables the original distribution of *coli*, and *I. A. C.* in water, milk, shell-fish, ice-cream, or other material to be estimated with considerable accuracy, that it involves less labour and is much more rapid, allowing results to be obtained in about four days, as compared with about 10 days for the routine method, and that it uses less media, dispenses altogether with plating, and is, therefore, considerably cheaper'.

At the suggestion of Prof. Wilson, an investigation was undertaken by us using the modified dilution method described by him in the Special Report No. 206 of the British Medical Research Council. The detailed procedure adopted by us in consultation with him is given below :—

- (a) Each sample of water was inoculated into MacConkey's bile-salt-lactose-neutral-red broth (*containing sodium chloride also in its composition)—50 c.c. of water into one tube, 10 c.c. into each of 5 tubes, 1 c.c. into each of 5 tubes and 0.1 c.c. into each of another 5 tubes. These were incubated for 48 hours at 37°C. The results were then read off and recorded.
- (b) At the end of 24 hours' incubation at 37°C. one tube showing acid and gas from each of the four dilutions was plated out on the MacConkey's bile-salt-lactose-neutral-red agar (also containing sodium chloride) and incubated at 37°C. for 24 hours at the end of which six colonies were picked off from each plate and confirmed on MacConkey's broth for production of acid and gas at the end of 24 hours at 37°C. All the strains which were positive were then put through the following tests : (1) methyl-red, (2) Voges-Proskauer, (3) citrate-utilization, (4) indole-formation, and (5) ability to produce acid and gas when grown in MacConkey's broth at a uniform temperature of 44°C. accurately maintained in a water-bath. The strains were then

* The original medium as described by MacConkey and as used by us at this Institute does not contain sodium chloride.

classified as *coli I*, *coli II*, intermediate-*ærogenes-cloacæ*, and irregulars.

(c) *At the end of 24 hours' incubation at 37°C. every tube showing acid and gas in the original 37°C. enrichment series was inoculated into (i) a MacConkey's broth tube and placed *without delay* in a water-bath at 44°C. to be incubated for 24 hours and (ii) a Koser's citrate broth tube and incubated at 37°C. for 48 hours. The results were then read off and the organisms classified as *coliform*, *fæcal coli*, and intermediate-*ærogenes-cloacæ*.

(d) All the tubes showing acid and gas in the MacConkey's broth tubes incubated at 44°C. were next plated out on MacConkey's agar and incubated at 37°C. for 24 hours. Two colonies were then picked from each plate and confirmed in MacConkey's broth for production of acid and gas. All strains found positive were submitted to the same five tests mentioned under (b) above, and classified as *coli I*, *coli II*, intermediate-*ærogenes-cloacæ*, and irregulars.

It will be seen from the above that the preliminary enrichment is still made at 37°C. (procedure *a*), that a differentiation of the *coliform* bacteria in these cultures into *coli* and *ærogenes* is made by adopting the procedure outlined in (c) and that the procedure detailed in (b) and (d) are intended to furnish additional confirmatory evidence in support of the classification by procedure (c).

Twelve samples of water derived from impounded surface reservoirs, wells, and rivers were thus examined by us in the first instance. They yielded a total of 416 cultures by the two methods (218 in the 37°C. enrichment series and 198 in the 44°C. sub-culture series). These were classified into *coli*, *I. A. C.*, and irregulars, on the basis of the M. R., V. P., citrate, and indole tests. But when the test for their ability to grow at 44°C. in MacConkey's broth (Wilson's method IV) came to be applied, 62 out of a total of 98 *ærogenes*-like cultures in the 37°C. enrichment series and 61 out of a total of 88 *ærogenes*-like cultures in the 44°C. sub-culture series were found to grow at 44°C. (*vide* Table I).

TABLE I.

Types.	Total number of cultures.	Number growing at 44°C.	Percentage to total.
37°C. Enrichment series.			
<i>Coli I</i>	82	75	91.5
<i>Coli II</i>	3	3	100.0

* Tubes showing acid and gas only at the end of 48 hours (but not at 24 hours) at 37°C. were also subjected to these two tests after 48 hours and the organisms classified. Similarly, tubes inoculated from the 37°C. series and found negative or doubtful after 24 hours at 44°C. were incubated for a further period of 24 hours at 44°C. and such as were positive after 48 hours were also plated out and the organisms classified.

TABLE I—concl'd.

Types.	Total number of cultures.	Number growing at 44°C.	Percentage to total.
<i>Ærogenes-cloacæ</i> I	98	62	63.3
<i>Ærogenes-cloacæ</i> II	1	1	100.0
<i>Intermediate</i> I	9	7	77.7
<i>Intermediate</i> II	3	3	100.0
<i>Irregulars</i>	22	15	68.1
44°C. Sub-culture series.			
<i>Coli</i> I	81	75	92.6
<i>Coli</i> II	1	1	100.0
<i>Ærogenes-cloacæ</i> I	88	61	69.3
<i>Intermediate</i> I	4	2	50.0
<i>Irregulars</i>	24	19	79.1

It will be seen that from 60 to 70 per cent of the *ærogenes*-like organisms isolated from water possessed the ability to produce acid and gas in 24 to 48 hours in MacConkey's broth at a temperature of 44°C. accurately maintained in a water-bath. This finding is contrary to those of Wilson (*loc. cit.*) and Bardsley (*loc. cit.*). They found comparatively few *ærogenes*-like organisms (M. R. —, indole —, citrate +, V. P. +) which possessed the ability to grow at 44°C. in MacConkey's broth, and these were placed in a group as irregular VI mainly on the basis of the 44°C. fermentation test. In attempting to express our results in terms of the probable number of the *coli* and *ærogenes* organisms, on the basis of Wilson's schematic classification, we found that the *coliform* and *I. A. C.* counts were practically identical in every case (*vide* Table III) showing that *I. A. C.* were present in all the samples and that Wilson's method IV was not, therefore, specific in our hands in differentiating the *coli-ærogenes* organisms without further elaborate tests. Wilson has apparently considered all the organisms which produce acid and gas at 44°C. to belong necessarily to the *coli* type. In our series organisms isolated

from tubes giving acid and gas at 44°C. resolved themselves, on further differentiation, into either *coli* or non-*coli* (*vide* Table II).

TABLE II.

Sample number.	Total number of organisms at 44°C.	Percentage of <i>coli</i> .	Percentage of non- <i>coli</i> .
1	8	62.5	37.5
2	24	4.2	95.8
3	23	47.8	52.2
4	24	72.0	28.0
5	26	30.2	69.8
6	11	82.0	18.0
7	26	19.2	80.8
8	4	25.0	75.0
9	16	31.2	68.8
10	2	100.0	0.0
11	27	51.8	48.2
12	7	57.0	43.0

It should be noted, however, that the tubes showing acid and gas in MacConkey's broth at 44°C. were sub-cultured on MacConkey's agar and incubated at 37°C. If *aerogenes-cloacae* organisms were present (not necessarily growing) in the broth cultures at 44°C. they will show up in isolation on the subsequent plate culture at 37°C. This factor might account to some extent for the findings recorded in the above table. It is not clear from the milk report of Wilson *et al.* how this limiting factor was overcome in their work. The only reference which we could find in the Report is at page 163 where it is stated 'At 44°C. practically all indole positive

strains of *B. coli* grew well and produced gas. *No strains of aerogenes produced gas at this temperature and very few even grew at all* (the italics are ours).

TABLE III.

Number of sample.	Number of MacConkey's tubes showing acid and gas at 37°C.	Number of tubes in column 2 which were positive by citrate test.	Number of tubes in column 2 showing acid and gas in sub-cultures at 44°C.	NUMBER OF TUBES IN COLUMN 4 SHOWING ON SUB-CULTURE		
				<i>Coli</i> and non- <i>coli</i> .	<i>Coli</i> alone.	Non- <i>coli</i> alone.
1	2	3	4	5	6	7
1	9	9	4	1	2	1
2	15	14	14	1	0	13
3	16	16	*12/15	4	4	4
4	16	16	14	3	9	2
5	17	17	*14/16	3	3	8
6	14	14	*6/8	0	5	1
7	18	18	14	5	1	8
8	5	5	2	2	0	0
9	10	10	9	1	2	6
10	6	6	*1/2	0	1	0
11	15	15	15	2	6	7
12	6	6	*4/5	0	2	2
TOTAL ..	147	146	109	22	35	52

*Note.—Although the actual number of tubes showing acid and gas at 44°C. were 15, 16, 8, 2 and 5 in these cases, only 12, 14, 6, 1 and 4 tubes respectively yielded *coliform* organisms in sub-cultures on MacConkey's agar plates at 37°C.

The above table clearly shows that 146 out of 147 positive tubes from the enrichment (37°C.) series contained citrate utilizers of the *colon* group (*aerogenes-cloacæ*) either alone or in combination with *coli*, that only 109 of them showed organisms capable of growing in sub-culture at 44°C. and that 52 of these 109 tubes (47.7 per cent) contained only non-*coli*. Only 32 per cent of the tubes contained typical *B. coli*. Even if the 22 tubes showing a mixture of *coli* and non-*coli* were added to this number, the total would still be only 57 tubes out of a total of 109

(52·3 per cent) in favour of the typical *B. coli*, for detecting which this test is deemed specific.

In view of the contradictory findings obtained by us, Prof. Wilson was requested to study our detailed results. While agreeing with our findings, he considered it desirable that we should check up the various details of the technique employed by us, with a view to eliminating possible sources of error or deviation. On a careful re-check, our technique was found to be accurate in every detail. Prof. Wilson was, therefore, furnished at his request with 24 strains of *coliform* organisms (*coli* and *aerogenes*) isolated by us from water. After subjecting these cultures to several tests in order to determine whether our results were vitiated to any extent by synergic action of two organisms working together, he informed us that his attempts to show this were unsuccessful and that the cultures behaved in his hands in the same manner as in ours and that, therefore, 'our main contention, viz., that some strains of *Bact. aerogenes* isolated from Indian waters produce acid and gas in MacConkey's broth at 44°C., appeared to him to be perfectly correct'. He concluded by saying 'Clearly, the 44°C. MacConkey's test cannot be relied upon in India for the differentiation of faecal from non-faecal *coliform* organisms, unless it can be shown that irregular VI (*aerogenes* which grew at 44°C.) is a natural inhabitant of the Indian intestine'.

Work on this aspect of the problem is in progress.

SUMMARY.

The methods recommended in Medical Research Council Special Report Series No. 206 for estimating the numerical presence of *coliform*, faecal *coli*, and intermediate-*aerogenes-cloacæ* organisms respectively in milk, etc., have been tried out on twelve samples of Madras waters. The results indicate that these methods may not be applicable to tropical waters. About 50 per cent of *aerogenes*-like organisms as isolated from these samples have been shown to produce acid and gas in MacConkey's broth at 44°C.

CONCLUSION.

It would, therefore, appear that the MacConkey's test at 44°C. can be considered sufficiently specific for differentiating *coli* into faecal and non-faecal types, *only* if it can be proved that *aerogenes*-like organisms in water capable of growing at 44°C. are normal intestinal organisms in India.

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LONGEVITY OF COLIFORM ORGANISMS IN WATER STORED UNDER NATURAL CONDITIONS.

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CLEMESHA's study of faecal organisms in water and faeces carried out in this Institute in 1908-1909 had special reference to the effect of exposure to direct sunlight. He divided *coliform* organisms found in faeces and water into three classes based on the capacity of the organisms to resist the action of sunlight. He claimed to have established that 'in any naturally occurring mixture of micro-organisms, a certain proportion (how great a proportion we have at present not much idea) of one or more species will be found to survive the others when the mixture is exposed to prolonged sunlight. But it is with this resistant residuum that the sanitarian in India is most frequently called upon to deal. Hence the great importance and utility of this classification'. This classification has been regularly applied at this Institute since 1908. The more recent tests based on hydrogen-ion concentration and citrate utilization have also been applied since 1921 for purposes of correlating the older classification of Clemesha with the more recent grouping.

Webster (1934) carried out some tests in this Institute and recorded his observations on the period up to which different strains of lactose fermenters and streptococci could be recovered from the Winchester quart bottles full of sterilized, distilled and river water infected with pure cultures and stored in the Laboratory. It was found that *B. schæfferi* (an organism closely allied to *B. coli communis*) was found to survive for 62 days in stored distilled water and for 34 days in stored river water and that *B. neapolitanus* (also a member of the true *coli* group) lived for 74 and 54 days respectively and when a mixture of three organisms belonging respectively to Clemesha's classes I, II, and III was similarly tested, the class I organisms (*B. coscoroba*) survived for 59 and 45 days, class II (*B. 67*) for 56 and 67 days, and

One organism of this type was isolated last on 10th August, 1938, and none thereafter. The heavy rainfall of about 5 inches (3·2" in one day, 21st August, 1938) did not, however, affect the results (no class I type was found), showing that the water in the reservoir is not liable to adventitious pollution being washed into it by the rain.

Applying the modern classification to this series, we find that 34 out of 41 samples showed the presence of typical *B. coli*; 237 out of 405 *coliform* bacteria isolated (58·5 per cent) were found to be of the true *B. coli* type. There was no consistent or progressive reduction in the numerical strength of these forms, with increased exposure to sunlight or storage.

DISCUSSION.

From the results of the findings recorded above, it would appear that organisms of Clemesha's class I are not killed out under the influence of sunlight, in anything like the very brief period claimed by Clemesha as being sufficient to ensure their disappearance under natural conditions. When it is remembered that the ubiquitous *B. vesiculosus* is present in faeces, cow-dung and water with remarkable constancy, that Clemesha found it to be resistant to the action of sunlight and storage for considerable periods and that it is really a member of the true *B. coli* group (M. R. +, V. P. —, citrate —, indole +) under the modern scheme of classification, the application to the sanitary assay of potable waters, of a classification of *coliform* bacteria based on their powers of resistance to sunlight and storage would appear to be of little or no value. In the two series of experiments recorded by us, *B. coli communis* (Clemesha's class I) was found to survive for a considerable time and to show no appreciable reduction in numbers during at least the first 4 or 5 weeks of exposure to sunlight and storage.

B. lactis aerogenes (an organism of Clemesha's class II) has generally come to be accepted as rare in faeces, but preponderant in soil, grains, etc., so much so that its presence in water is not viewed with the same degree of suspicion as *B. coli*. There are, however, some water bacteriologists who do not take the same lenient view of its presence in water on the score of its being very often the offending organism in urinary infections. We have reason to believe that even amongst the *aerogenes* forms, there are some which are truly faecal, although others may be non-faecal. This view of ours is based on the behaviour of *aerogenes* cultures from water grown in MacConkey's broth at 44°C. Confirmatory work is in progress using *aerogenes* cultures isolated from faeces.

We are thus left with only one fairly reliable index for the assay of potability which is the intensity of pollution revealed by the *coliform* count which gives a quantitative estimate of the *coliform* organisms in water by the routine enrichment method.

SUMMARY.

1. The present note relates to experiments carried out with Adyar river water stored in open masonry reservoirs 30' × 30' × 6' for four months during the monsoon season and for six months during the hot weather.

2. *Coliform* organisms of Clemesha's class I (susceptible to sunlight and storage) were isolated in 32 out of 46 samples between November 1937 and March 1938, and 216 out of 564 cultures isolated belonged to class I.

3. During the hot weather, only 16 out of 41 samples showed their presence and 54 out of 405 cultures belonged to class I. Though reduced in number, the class I organism was isolated from the water after four months of storage and exposure to sunlight.

4. If the modern classification of *coliform* bacteria into *B. coli* and intermediate-*aerogenes-cloacæ* is applied to our results, 80 per cent of the organisms found during the hot weather belonged to the true *B. coli* type.

CONCLUSION.

In the present state of our knowledge of the *colon-aerogenes* group of organisms, it is not safe to form an estimate of the recency of pollution in water-supplies on the basis of the resistance of the *coliform* organisms to sunlight and storage.

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THE TECHNIQUE OF THE METHYL-RED AND VOGES-PROSKAUER TESTS USED IN THE BACTERIOLOGICAL ANALYSIS OF WATER.

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THE distribution in nature of lactose-fermenting bacilli has been extensively studied during the past 30 years and it has been found that, whereas in human and animal faeces the *B. coli* comprise 90 per cent of the whole, in soil, cereals, plants, etc., the *B. aerogenes* predominate. While *B. coli* are believed to die out rather quickly, *B. aerogenes* are credited with the power to survive outside the animal body. The presence of *B. aerogenes* in water is consequently held to be less indicative of recent excremental pollution than that of the *B. coli*. In the bacteriological analysis of water, the differentiation of the lactose fermenters present into *coli* and *aerogenes* is, therefore, considered essential. The first clear cut distinction between *coli* and *aerogenes* is to be found in the classical work of MacConkey (1905, 1908) who defined the *aerogenes* type as saccharose positive, dulcitate negative, indole negative, and V. P. positive. These characters continue to be used as the basic factors in differentiation. A few additional tests have, however, been added during recent years, e.g., the methyl-red and citrate tests (Rogers *et al.*, 1914, 1918; Koser, 1923). Five tests are now usually considered sufficient (indole, M. R., V. P., citrate, gelatin liquefaction) for differentiation purposes and the Report of the British Ministry of Health (1934) has classified the *colon-aerogenes* group into seven common types on the basis of these five tests. The procedures adopted in carrying out the five tests are detailed in that report.

The present note relates to the technique of two of these five tests, viz., the M. R. and V. P. tests. Smith (1895) noted that the activity of gas production in

sugar media by *B. aerogenes* and *B. cloacæ* was greater than that of *B. coli*, that the ratio of CO_2 to H_2 was higher and that the final degree of acidity lower. This is the basis of the methyl-red test evolved by Rogers *et al.* (1918) which is performed in the following manner :—

Buffered glucose-phosphate broth is inoculated with a loopful of the culture and incubated for three days at 37°C . Five drops of a 0.04 per cent solution of methyl-red are then added to the culture. A magenta-red colour is considered positive, while a definite yellow colour is negative. Intermediate grades of colour indicate at best a doubtful result.

The Voges-Proskauer test (V. P. test).—Used since 1898 to differentiate between *B. coli* and *B. aerogenes*, this is a test for acetyl-methyl-carbinol [$\text{CH}_3\text{CH}(\text{OH})\text{CO}\cdot\text{CH}_3$] which, in the presence of caustic alkali and air, becomes oxidized to diacetyl-methyl-carbinol ($\text{CH}_3\text{CO}\cdot\text{CO}\cdot\text{CH}_3$) which, in turn, reacts with a substance in the broth to produce a pink fluorescence. This substance is believed to be arginine possessed of a guanidine grouping [$\text{NH} : \text{C}(\text{NH}_2)\cdot\text{NH}\cdot\text{R}$] (Barritt, 1936). This test is usually performed by inoculating a second tube of glucose-phosphate broth with the culture, incubating it for three days at 37°C . and adding to it 5 c.c. of a 10 per cent solution of sodium hydroxide. The result can be read only after the tube has stood for 12 to 24 hours at room temperature or after heating the mixture to near boiling point for 30 minutes. A pink fluorescence denotes a positive and no colour a negative result.

As performed above, the V. P. test is often found to give vague indefinite reactions and various modifications have, therefore, been proposed from time to time, all directed towards intensifying the oxidation of acetyl-methyl-carbinol. Thus, Levine, Weldin and Johnson (1917) used hydrogen peroxide, barium peroxide, and potassium dichromate; Bedford (1929), sodium peroxide; Werkman (1930), ferric chloride; O'Meara (1931), creatine; Leifson (1932), a mixture of copper sulphate ammonia, and soda; and Barritt (*loc. cit.*), α -naphthol.

Of these, only O'Meara's modification has been considered to be a definite improvement, in that the colour change is markedly intensified; but even with this, indefinite reactions were still obtained, particularly in dealing with the organisms of the 'intermediate' group. Barritt's modification is claimed to overcome this defect by giving a definite and more rapid intensification of the colour change, with a significant increase in sensitiveness. The technique of this modification as given by Barritt is as under :—

After three days' incubation at 37°C ., one c.c. of the broth culture is placed in a $6'' \times \frac{3}{4}''$ test-tube to which are added 0.6 c.c. of α -naphthol (5 per cent alcoholic solution) and 0.2 c.c. of KOH (40 per cent solution). Equally good results are obtainable with 0.5 c.c. of a 6 per cent solution of α -naphthol and 0.5 c.c. of a 16 per cent solution of KOH. After shaking the tubes to mix the contents, positive reactions appear as a pink colour in 2 to 5 minutes—this intensifies on further standing.

We have applied this as well as other modifications in the routine analysis, during the past 12 months, of over 600 samples of water which yielded upwards of 3,000 cultures of *coliform* bacteria. Barritt's modification was found to yield a much more intense colour change, to be more delicate and sensitive than the ordinary

V. P. test and to yield definitely positive reactions in some cases where the ordinary V. P. test and the other modifications yielded a negative or only a vague and indefinite red colour change. The positive results obtained with Barritt's technique correlated perfectly with the M. R. and indole tests. Barritt's claim to greater sensitiveness and specificity was thus established. It will be seen that, in the routine performance of the M. R. and V. P. tests, two tubes of glucose-phosphate broth are inoculated with a loopful of each of the cultures under test, one for the M. R. and the other for the V. P. test. Some would appear to use only one tube of glucose broth for culturing and divide it into two portions for carrying out the two tests after three days' incubation at 37°C.

In the course of our tests with Barritt's modification, we happened one day by accident to add the V and P reagent (α -naphthol) to the same series of cultures which had already received the M. R. test reagent. Thereupon we added KOH also to these tubes and found that the V and P reaction developed just as well. Encouraged by this finding, we duplicated our cultures and systematically tried the method of using the same set of cultures for both tests. One set of culture tubes (glucose-phosphate broth) after three days' incubation at 37°C. received the M. R. reagent and after recording the result, α -naphthol and KOH were added to the same cultures, shaken and results recorded. Another set of the same cultures was dealt with in the usual manner, i.e., adding M. R. and V. P. reagents in separate tubes of cultures. The results of both sets were identical in 1,480 cultures and there was not a single instance where there was variation or vagueness in the colour change in the two sets thus compared.

The hydrogen-ion concentration produced by the *coliform* bacteria when grown in suitably buffered glucose broth is the property utilized in the methyl-red test. Cultures of *B. coli* elaborate acid until the lethal concentration in the pH zone (near pH 5.0) is reached and remain practically constant thereafter, while cultures of *B. aerogenes* destroy *all* the carbohydrate present in the media before reaching this zone. A reversal of the reaction then occurs, the pH in the two cases moving further apart, as the period of incubation increases. Thus, the *aerogenes* group forms *initially* a lower hydrogen-ion concentration but later a reversion towards neutrality occurs by the production of carbonates. Methyl-red turns yellow at pH 6.5.

The addition of the methyl-red indicator to the broth cultures cannot, therefore, interfere with the subsequent oxidation of the acetyl-methyl-carbinol into diacetyl when α -naphthol and KOH are added. This has been borne out by our results on about 1,500 cultures. We, therefore, consider that the procedure outlined by us above simplifies the technique and saves time and labour.

It would, therefore, appear to be justifiable to advocate the use of a single glucose-phosphate broth tube for both the tests. This would not only result in a saving of media, glassware, time and energy, but also ensure identical conditions as regards initial inoculum and final strength of the culture used for the tests after three days' incubation.

SUMMARY.

1. Barritt's modification of the Voges-Proskauer test, which makes use of α -naphthol in addition to KOH has been applied (along with several other

modifications which are also claimed to be more sensitive than the original V. P. test) on 3,000 cultures derived from over 600 samples of water.

2. Barritt's claim to greater specificity and sensitiveness has been established.

3. The use of a single glucose-phosphate broth tube for each culture, on which to carry out first the M. R. test and then the V. P. test (Barritt's modification) instead of two separate broth tubes is advocated. Controlled tests have shown that this may be done successfully without the results being vitiated in any manner.

4. The above procedure conduces to economy and simplifies the technique.

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EFFECT OF TRYPTIC DIGESTION ON THE TOXICITY AND THE ANTIGENICITY OF THE TETANUS TOXIN-BROTH.

BY

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IN recent years much work has been done on the separation of immunologically active fractions from the bodies of a number of different species of bacteria with excellent results. Recently, Ramon, Boivin and Richou (1937) obtained trichlor-acetic acid precipitates of the diphtheria and tetanus toxin-broth and compared their antigenic power with that of the original toxin-broth from which the precipitates were obtained, and found almost equal results with both. The method was adapted from that of Boivin and Mesrobian's (1933) trichlor-acetic acid extraction of *Bact. ærtrycke*.

This report encouraged the trial of the method of Raistrick and Topley (1934) for the purification and concentration of the toxin-broth. As a preliminary it was necessary to find out whether the tryptic digestion itself had any deteriorating action on the antigenicity of toxin-broth. In the case of the diphtheria toxin-broth, Brandwijk and Tasman (1937) found that tryptic digestion diminished its toxicity to a remarkable extent. Though their work did not make it clear whether that had led to deterioration of the antigenicity too, it was thought better to try the method in the case of tetanus toxin-broth, on which the effect of tryptic digestion was unknown.

TECHNICAL.

Production of tetanus toxin-broth.

Cl. tetani was grown in veal peptone-broth of the following composition :—

Veal	750 g.
Peptone (Witte)	10 g.
Sodium chloride	5 g.
Distilled water	1 litre.

Peptone was added to the veal and both simmered at 68°C. for 20 minutes in a water-bath. Sodium chloride was added after filtration. The final pH of the broth was 7.4.

Narrow-mouthed, amber-coloured, reagent bottles of two-litres capacity were filled with the sterilized broth almost up to the neck. The bottles were fitted with sterile rubber-stopper and incubated for 48 hours before inoculation to prove freedom from contamination. Five c.c. of the liquid portion of a 24 hours culture of *Cl. tetani* in cooked meat medium were put into each bottle with aseptic precautions. The bottles were incubated at 36°C. to 37°C. for 12 days and then filtered through Seitz E. K. pads under a negative pressure of about 20 cm. of mercury.

Determination of the minimal lethal dose.

The minimal lethal dose was determined in guinea-pigs of 350 g. weight by subcutaneous injection of 1 c.c. of each dilution and noting which caused deaths in about 96 hours.

The particular sample of toxin-broth which was used for the experiment had a minimal lethal dose in 1/20,000 c.c. when determined just after filtration.

The toxin-broth was stored at 0°C. to 2°C. for about a month for 'maturing'. At the commencement of the experiment the minimum lethal dose was found to be 1/9,000 c.c. of the toxin-broth.

Tryptic digestion of toxin-broth.

In two sterile flasks of about 500 c.c. capacity, 400 c.c. of the toxin-broth were put in each. The amount of NaOH required to adjust the pH in each flask to about 8.4 (colorimetrically) was determined and added to each. 0.4 g. trypsin (Merck) weighed in a sterile crucible was added to one of them marked A and the other marked B was left as a control. Twenty c.c. of toluol were layered over each and a sterile rubber-stopper applied. The flasks were incubated at 36°C. to 37°C.

To flask A, 0.3 g. trypsin was added on the 3rd day (at the end of 48 hours), 0.2 g. on the 6th day (at the end of 120 hours), and 0.1 g. on the 8th day (at the end of 168 hours), to make sure that the process of digestion did not stop due to the want of a sufficient amount of trypsin.

EXPERIMENTAL.

Determination of the correlation between the degree of toxicity and the degree of digestion.

From each of the flasks A and B, 25 c.c. of the toxin-broth were taken out on the 3rd, 4th, 5th, 6th, 7th, 8th and the 10th days. Of this amount, 20 c.c. were required for Sørensen's formol titration, and the remaining amount was used for making the dilutions for injecting the guinea-pigs in order to find the minimal lethal dose of the treated and untreated toxin-broth on those days. The minimal

dose was taken as the criterion of toxicity and the rise in amino-nitrogen content as the criterion of digestion.

A. *The degree of toxicity.*—For the determination of the degree of toxicity, the dilutions of each day's samples from flasks A and B used for injecting the guinea-pigs were selected according to the experience of the preliminary experiments. They were made in saline and were as follows :—

Dilutions made from flask A.

Samples of	Grades of dilution.				
	1	2	3	4	5
3rd day ..	1/2,000	1/3,000	1/4,000	1/5,000	1/6,000
4th day ..	1/1,000	1/1,500	1/2,000	1/2,500	1/3,000
5th day ..	1/500	1/750	1/1,000	1/1,250	1/1,500
6th day ..	1/250	1/375	1/500	1/625	1/750
7th day ..	1/125	1/200	1/250	1/325	1/375
8th day ..	1/50	1/100	1/125	1/175	1/200
10th day ..	1/25	1/50	1/75	1/100	..

Dilutions made from flask B.

Samples of	Grades of dilution.		
	1	2	3
3rd day ..	1/6,000	1/7,000	1/8,000
4th day ..	1/6,000	1/7,000	1/8,000
5th day ..	1/5,000	1/6,000	1/7,000
6th day ..	1/5,000	1/6,000	1/7,000
7th day ..	1/4,000	1/5,000	1/6,000
8th day ..	1/4,000	1/5,000	1/6,000
10th day ..	1/3,000	1/4,000	1/5,000

Each dilution was injected subcutaneously in 1 c.c. amounts into one guinea-pig only of about 350 g. weight. The time of death of the guinea-pigs dying of tetanus was noted.

The results are recorded in Tables I and II :—

TABLE I.

Hours of death of guinea-pigs injected with different dilutions (loc. cit.) of the contents of flask A.

Samples of	Dilution number.				
	1	2	3	4	5
3rd day ..	67	67	91	98	121
4th day ..	94
5th day ..	91	97
6th day ..	67	91	91	99	..
7th day ..	95	94	115
8th day ..	91	115
10th day

TABLE II.

Hours of death of guinea-pigs injected with different dilutions (loc. cit.) of the contents of flask B.

Samples of	Dilution number.		
	1	2	3
3rd day ..	91	91	97
4th day ..	91	95	102
5th day ..	91	99	115
6th day ..	91	115	..
7th day ..	75	96	115
8th day ..	101	93	..
10th day ..	91	115	..

The times of death of the guinea-pigs in hours are only approximate, especially the numbers such as 67, 91, and 115, which denote the time of death to be between 55 and 67 hours, 79 and 91 hours, and 103 and 115 hours respectively since the injections were given at 1 p.m. and the animals were under observation only between 8 a.m. and 8 p.m. everyday. A guinea-pig found dead in the morning might have died any time between 8 p.m. and 8 a.m. The italicized hours of death represent those due to dilutions containing a minimal lethal dose.

B. *The degree of digestion*—was determined by the estimation of the increase in amino-nitrogen content as determined by Sørensen's method of formol titration, compared with the amino-nitrogen content of the undigested control. The result is shown in Table III :—

TABLE III.

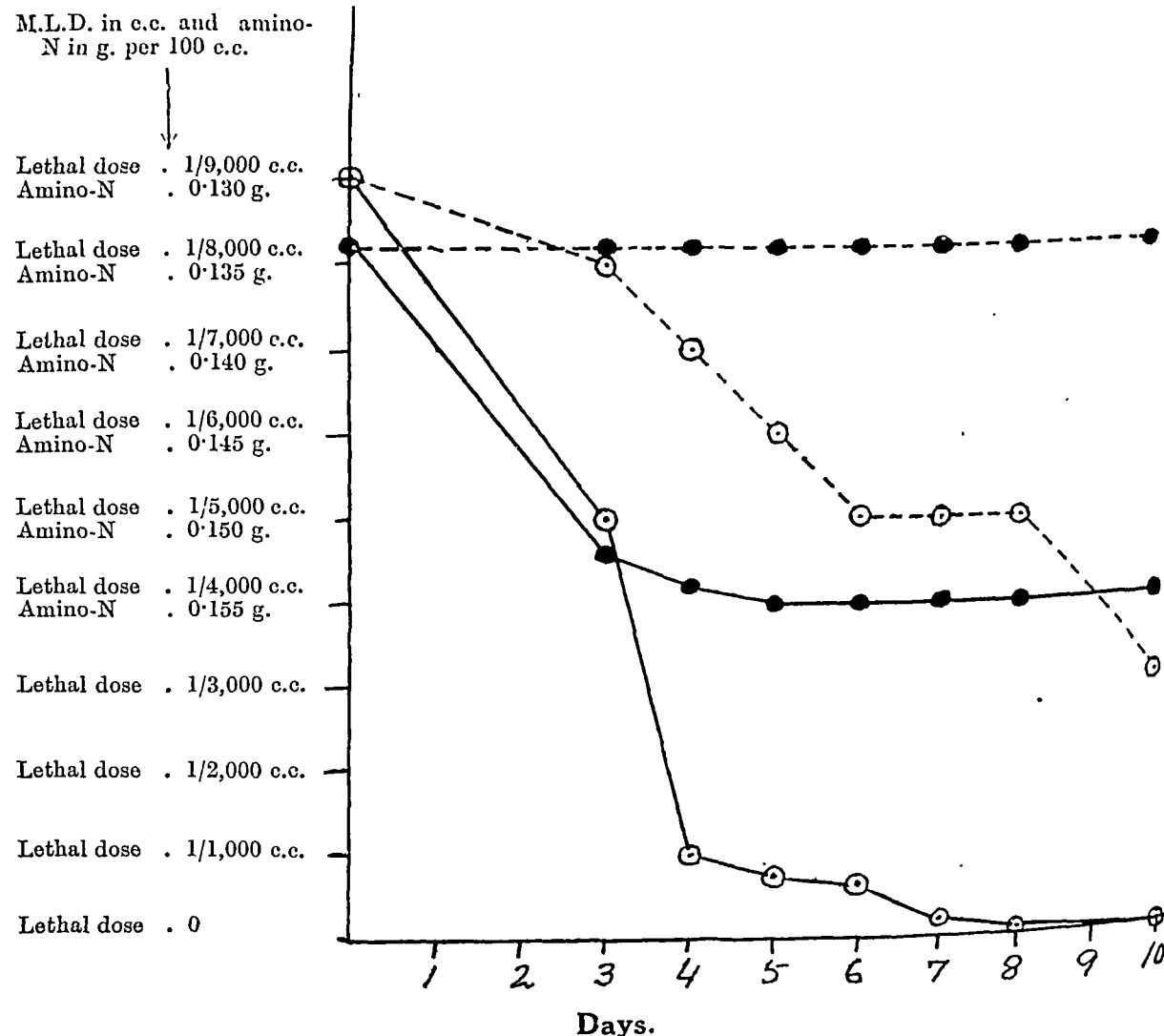
Amino-nitrogen percentages of digested and undigested toxin-broth.

Samples of	Digested toxin-broth (flask A).	Undigested toxin-broth (flask B).
3rd day ..	0.152	0.134
4th day ..	0.154	0.134
5th day ..	0.155	0.134
6th day ..	0.155	0.134
7th day ..	0.155	0.134
8th day ..	0.155	0.134
10th day ..	0.155	0.134

C. The correlation of the degree of toxicity with the degree of digestion.—

GRAPH.

Showing the correlation of the degree of toxicity and the degree of digestion of toxin-broth.



○—○ = Toxicity of the digested toxin-broth.

○---○ = Toxicity of the undigested toxin-broth.

●—● = Change in the amino-nitrogen content of the digested toxin-broth.

●---● = Change in the amino-nitrogen content of the undigested toxin-broth.

Determination of antigenicity.

Flask A was stored at 0°C. to 2°C. and in order to diminish the toxicity of the undigested toxin-broth 0.5 c.c. commercial formalin was put into flask B, which was then incubated at 36°C. to 37°C. for seven days. This was necessary, because otherwise it was not possible to immunize the rabbits with such a toxic substance. After incubation, flask B also was stored at 0°C. to 2°C.

Rabbits of about 2 kg. weight were selected for immunization with the toxin-broths of flasks A and B—two rabbits for each sample. They received two injections every week, both intravenously and subcutaneously—the total number of injections were twenty-five in the course of three months. The graduation of the doses was as follows :—

Intravenous.—0.2 c.c., 0.4 c.c., 0.6 c.c., 0.8 c.c., 1.0 c.c.—the last one continued till the total number was twenty-five.

Subcutaneous.—0.2 c.c., 0.4 c.c., 0.6 c.c., 0.8 c.c., 1.0 c.c., 1.5 c.c., 2 c.c., 2.5 c.c., 3 c.c., 3.5 c.c., 4 c.c., 4.5 c.c., 5 c.c.—the last one continued till the total number was twenty-five.

The animals were bled from the heart seven days after the thirteenth injection and also again seven days after the last injection. The serum was titrated against an L_{+} dose of the test toxin determined against 0.1 International unit of the standard serum, with the following result (Table IV) :—

TABLE IV.

Comparative antigenicity of digested and undigested toxin-broth.

	Rabbit number.	TITRE IN INTERNATIONAL UNITS PER C.C. OF SERUM OF :—	
		1st bleeding.	2nd bleeding.
Rabbits immunized with the digested toxin-broth. {	1	<0.1	<0.1
	2	<0.1	<0.1
Rabbits immunized with for molized undigested toxin-broth. {	3	>3 <5	>12.5 <15
	4	5	>15

DISCUSSION AND CONCLUSION.

It appears from the study of the correlation between the degree of toxicity and the degree of digestion, that the increased fall in toxicity of the digested, in

comparison to that of the undigested, toxin-broth is due to the tryptic digestion. The temperature of incubation also caused some deterioration of the toxicity. The loss of antigenicity together with the fall in toxicity suggests that the tryptic digestion caused a sufficient disturbance in the structure of the toxin molecule or its nearest environment to destroy both of its properties. This might possibly be due to the protein nature of the toxin molecule or its closest associate, as is also suggested by the experiments of Eaton (1936) on the purification of tetanus toxin.

SUMMARY.

Experiments are described the results of which show that the tetanus toxin-broth loses its toxicity and antigenicity when digested with trypsin.

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A DIET SURVEY OF FAMILIES WITH LEPROSY.

BY

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THERE has recently been a good deal of speculation about the possible relation between leprosy and malnutrition. In India, leprosy is considerably more common in Madras and Bengal, in which rice is the staple cereal, than in North India where the diet is based on whole wheat, and milk is consumed by most of the population. The association of leprosy with poverty has long been recognized. Few scientific investigations designed to throw light on the problem have, however, been undertaken, and the factors underlying the epidemiology of leprosy remain obscure.

For some time it has been known that Saidapet, a suburb of Madras, is an area of high leprosy endemicity, though it is not certain that the disease is more prevalent in this district than in other parts of the city or elsewhere in South India. Saidapet is largely populated by weavers who work in their homes, and unquestionably the economic level of the suburb is extremely low. A house-to-house survey of leprosy in this area, carried out by Dr. R. G. Cochrane, Medical Officer of the Tirumani Leper Settlement, Chingleput, has revealed the high incidence of about 50 cases per 1,000 inhabitants.

DIET SURVEY.

In collaboration with Dr. Cochrane and his assistants, a diet survey of 14 families, comprising of 83 people, was undertaken in the area. The families were not deliberately chosen from among those containing the greatest number of lepers; the chief criterion of selection was the degree of co-operation obtainable. Subsequently, however, it was found that there were cases of leprosy in all the families surveyed, with one exception.

A Diet Survey of Families with Leprosy.

The duration of the survey, which was carried out in January and February, was as follows:—

8 families for 20 days.
4 " 19 "
1 family " 18 "
1 " 15 "

The survey was carried out under our supervision and that of the Medical Officer attached to the Saidapet Leprosy Clinic. Most of the actual weighing of foodstuffs was done by students of the Y. M. C. A. College of Physical Education, Saidapet. The usual technique of the Laboratories for the collection and working up of data was followed (Aykroyd and Krishnan, 1937). Mean intake of protein, etc., in the 14 families, and the range in the case of each factor, are given in Table I. Table II shows daily intake of various foodstuffs.

TABLE I.

Intake of protein, etc., per consumption unit per day.

	Mean intake.	Range.
Protein	42 g.	22 g. to 64 g.
Fat	22 g.	11 g. to 35 g.
Carbohydrate	355 g.	191 g. to 489 g.
Calories	1,790	960 to 2,350.
Percentage of calories from cereals ..	78	69 to 87.
Calcium	0.25 g.	0.10 g. to 0.44 g.
Phosphorus	0.94 g.	0.44 g. to 1.55 g.

Calorie intake was insufficient and the diet was particularly deficient in calcium. The chief ingredient was milled parboiled rice, consumed in insufficient quantities. Milled parboiled rice contains approximately 1.8 μ g. of vitamin B₁ per gramme. The daily amount of vitamin B₁ supplied by rice, the principal source of this vitamin in the diet, varied from 400 μ g. to 1,000 μ g. This amount is enough to prevent beri-beri but is below the standard usually suggested as representing adequate intake. Badger and Sebrell (1935) observed that in rats on a diet deficient in vitamin B₁ the incubation period of rat leprosy was appreciably shorter than in rats on a well-balanced diet. How far rat leprosy is analogous to human leprosy is not clear.

Intake of vegetables, fish, and meat was a little higher than that observed in previous surveys in South India. On the whole, however, the picture obtained is that of a group whose diet is very deficient in quantity and quality. It is no exaggeration to say that most of the families were half-starved when the survey was made.

TABLE II.
Intake of various foodstuffs. (Oz. per consumption unit per day.)

TABLE II.													
Intake of various foodstuffs. (Oz. per consumption unit per day.)													
Family number.	Number of persons in family.	Milled parboiled rice.	Other cereals.	Oils and fats.	Whole milk.	Fish.	Meat.	Eggs.	Pulses.	Green leafy vegetables.	Other vegetables.	Fruit.	
1	4	12.4	Nil	0.6	Nil	0.3	0.7	Nil	1.3	0.4	3.4	Nil.	
2	4	17.1	Nil	0.3	Nil	1.7	1.0	Nil	0.5	0.5	1.5	Nil.	
3	6	12.0	1.2	0.9	2.6	0.7	0.5	0.1	1.1	1.0	2.3	0.6	
4	4	14.3	Nil	0.5	Nil	Nil	0.6	Nil	0.8	0.1	3.7	Nil.	
5	6	11.8	Nil	0.5	Nil	0.5	1.2	0.2	0.8	1.2	2.9	Nil.	
6	5	18.1	Nil	0.4	Nil	0.5	0.9	Nil	Nil	0.3	2.0	Nil.	
7	5	12.9	Nil	0.4	Nil	1.0	0.3	Nil	0.2	0.4	0.9	Nil.	
8	6	15.4	0.1	0.4	0.6	0.1	0.6	Nil	1.5	0.4	4.6	0.3	
9	9	13.0	0.3	0.8	6.2	0.7	0.8	0.2	1.0	0.3	4.2	0.3	
10	3	18.2	Nil	0.4	0.2	2.0	2.0	0.3	0.7	0.8	3.6	Nil.	
11	8	19.8	Nil	0.5	Nil	1.0	Nil	0.1	1.1	0.3	4.0	Nil.	
12	6	7.5	Nil	0.3	Nil	Nil	0.2	Nil	0.9	0.2	1.7	Nil.	
13	8	16.6	Nil	0.5	6.0	0.4	1.3	0.2	0.7	0.4	2.0	Nil.	
14	9	9.5	Nil	0.3	Nil	0.7	0.6	Nil	1.6	Nil	1.9	0.2	

LEPROSY SURVEY.

Dr. Cochrane's investigation revealed a high incidence of leprosy in these families at the time of the diet survey. Leprosy was found in all the families except one. Out of the 41 adults examined, 7 were suffering from leprosy. Among 39 children, there were 18 definite and 6 suspected cases. The incidence of leprosy in the group was, therefore, 35 per cent.

The worst fed family in the group was No. 12; mean calorie intake in this family was only 955 per day. Of 6 individuals belonging to this family examined, 5 showed signs of leprosy. Family No. 14 had a daily calorie intake of only 1,300; 7 out of 11 examined were found to have leprosy. This family included two more members when the leprosy survey was made than at the time of the diet survey.

DISCUSSION.

No conclusion can be drawn from this investigation with regard to the rôle of malnutrition in leprosy. All that has been shown is that a group with a high incidence of leprosy consumed a very deficient diet. Calorie intake was lower than that observed in any survey previously carried out in South India. Further investigations are necessary in areas in which there is a high incidence of leprosy and elsewhere in order to throw light on the relation between diet and leprosy.

SUMMARY.

A diet survey of 14 families, 13 of which contained cases of leprosy, has been carried out. The diet was found to be deficient in quantity and quality.

ACKNOWLEDGMENTS.

We acknowledge gratefully the co-operation of Dr. R. G. Cochrane, Medical Officer, Lady Willingdon Leper Settlement, Chingleput, in this investigation. Acknowledgments are also due to Dr. Rajagopal, Medical Officer, Saidapet Leprosy Clinic, and to Mr. H. C. Buck, Principal, Y. M. C. A. College of Physical Education, Saidapet, and students of this institution for their willing co-operation.

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DIET SURVEYS IN THE NILGIRIS AND TRAVANCORE.

BY

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THE research programme of the Laboratories includes the carrying out of diet surveys in sample areas in various parts of India. Considerable progress has been made with this scheme and the results of a number of investigations have already been published.

THE PRESENT INVESTIGATION.

The survey in the Nilgiris was undertaken largely for the benefit of a class of Health Officers under training in the Laboratories, and was in the nature of a demonstration. The families, 16 in number comprising 90 individuals, were employed on a tea plantation and lived in lines closely adjacent to the Laboratories. The class gave practical assistance in the collection of data. The survey lasted one week in the month of June, the period of inquiry being somewhat shorter than in previous surveys. While the survey was undertaken primarily for teaching purposes, useful data were obtained which can justifiably be added to the mass of information now being collected about dietary habits in India. No other diet survey of tea plantation coolies has hitherto been carried out in South India.

The second survey, carried out in Oollannoor, Travancore, included nine families (47 persons). It was made in January and lasted 10 days. The actual weighing out of foods was carried out by students engaged in an economic inquiry in the district.

Mean intake of the various food factors and its range are shown in Table I, while Table II gives the mean daily consumption of the chief foodstuffs. The usual methods of calculation were followed (Aykroyd and Krishnan, 1937).

The diet of group I was deficient in quantity and quality. In connection with calorie intake it must be remembered that these families were living in a temperate and not a tropical climate. The insufficient calcium intake is particularly noticeable. A fairly high intake of vegetables was recorded, but leafy vegetables were not eaten by most families. Only one family had any milk during the period of inquiry. The diet may be described as about the usual for families of poorer classes in South India. Both adults and children appeared to be in a poor state of nutrition.

TABLE I.

Intake of calories, proximate principles, calcium, and phosphorus per consumption unit per day.

	GROUP I. (NILGIRIS).		GROUP II. (TRAVANCORE).	
	Mean intake.	Range.	Mean intake.	Range.
Protein	43 g.	33-64 g.	32 g.	26-50 g.
Fat	18 g.	10-30 g.	21 g.	3-41 g.
Carbohydrate	449 g.	317-534 g.	507 g.	323-717 g.
Calories	2,140	1,440-3,170	2,380	1,590-3,190
Percentage of calories from rice	82	73-89
Percentage of calories from tapioca.	34	22-54
Calcium	0.19 g.	0.11-0.31 g.	0.46 g.	0.23-0.75 g.
Phosphorus	0.94 g.	0.71-1.21 g.	1.00 g.	0.63-1.15 g.

TABLE II.

Mean intake of various foodstuffs. (Oz. per consumption unit per day).

	GROUP I (16 FAMILIES).		GROUP II (9 FAMILIES).	
	Oz.	Remarks.	Oz.	Remarks.
Milled parboiled rice ..	18	..	9	..
Tapioca	None	..	18	..
Pulses	1	..	1	..
Leafy vegetables	Consumed in very small amounts by three families.	..	Consumed by only one family.
Other vegetables	3	..	8	Yams formed 75 per cent of the intake.
Fresh whole milk	Intake negligible	..	Intake negligible.
Butter-milk; curds	None	..	2	..
Fruits	None	..	5	Banana and jackfruit.
Coco-nuts	0.1	..	0.8	..
Meat and fish	Consumed in very small amounts by five families.	..	Mean of four families 1.5 oz. per day. Other families did not consume meat and fish.
Oils	0.4	Mainly gingelly-oil	0.3	Coco-nut oil.

It is noteworthy that most of the families were in regular employment, the usual daily earning of tea plantation coolies being about 6 annas for a man and 5 annas for a woman. It is probable, however, that most families were in debt.

The Travancore group is of interest because tapioca is an important ingredient in the diet. This is reflected in the low protein intake; tapioca, being a root and not a cereal, contains only 1 to 2 per cent of protein. Intake of 'other vegetables' and fruit was fairly high in this group. The outstanding defect of the diet is its low protein content. Both children and adults in this district in which the survey took place are poorly developed and their general state of nutrition leaves much to be desired.

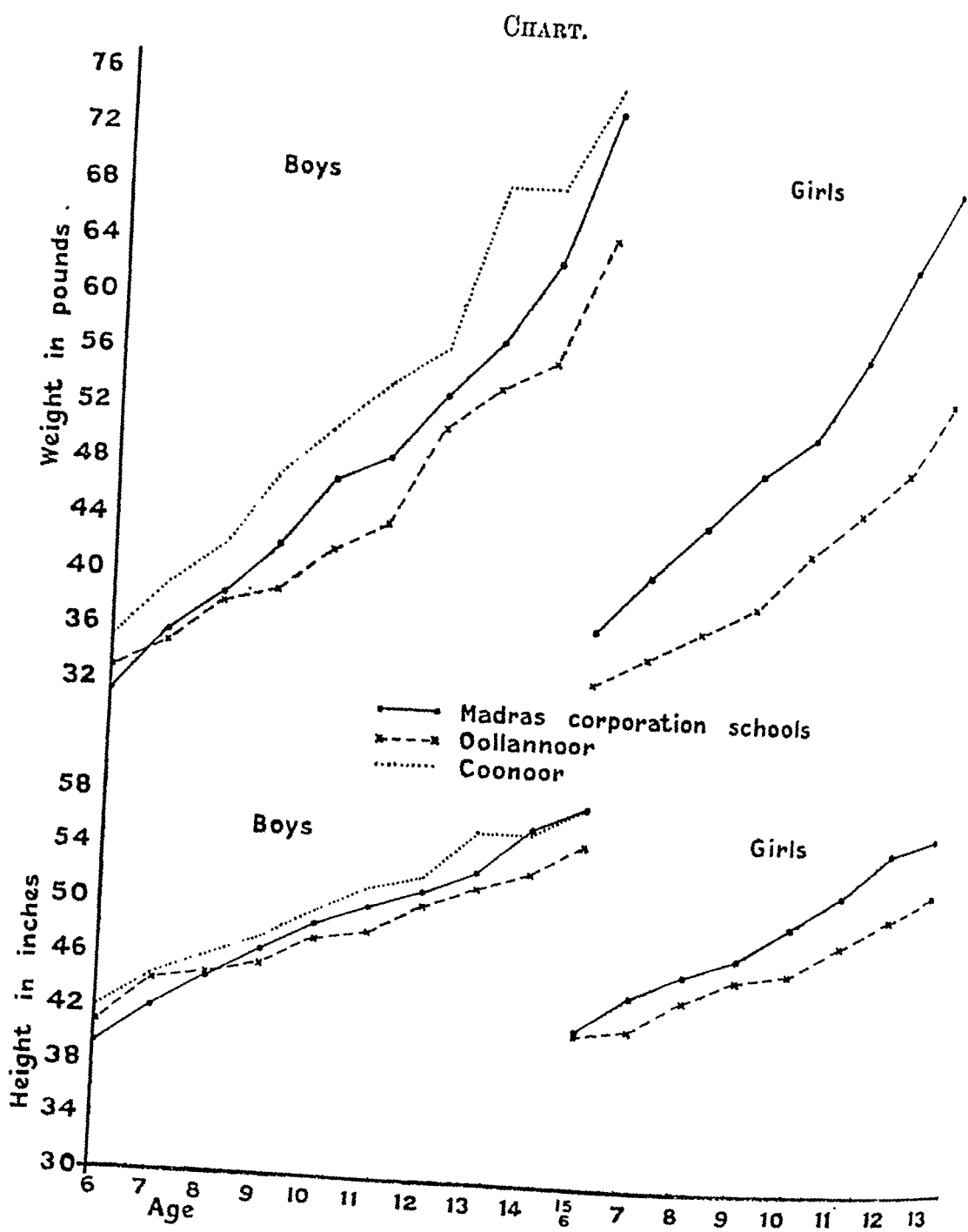
HEIGHT AND WEIGHT OF CHILDREN.

Two hundred and seventy-eight boys and 197 girls between the ages of 6 and 15 living in the neighbourhood of Oollannoor were weighed and measured. These children belonged to families living on a diet similar to that of families included in the survey. The means for each age group are shown in Table III. In the Chart the means in the group are compared with those of other groups in South India. One of these consists of boys attending schools in Coonoor (Nilgiris), belonging for the most part to families of the same economic status as the group of plantation labourers investigated, and consuming the same kind of diet. Figures for boys and girls in the Madras Corporation Schools were obtained from the Annual Reports of the Health Department, Corporation of Madras, 1930-1935.

TABLE III.

Mean weight and height for age of boys and girls in a tapioca eating district (Oollannoor).

Boys.				GIRLS.			
Age.	Number examined.	Average weight in lb.	Average height in inches.	Age.	Number examined.	Average weight in lb.	Average height in inches.
6	25	33	40.9	6	19	33	41.3
7	26	35	44.2	7	27	35	41.8
8	38	38	44.8	8	30	37	44.1
9	25	39	45.6	9	20	39	45.7
10	30	42	47.6	10	29	43	46.3
11	32	44	48.2	11	21	46	48.4
12	34	51	50.3	12	30	49	50.3
13	31	54	51.8	13	21	54	51.9
14	21	56	53.0
15	16	65	55.1



Mean heights and weights of children in Oollannoor, Travancore, compared with those of children in other parts of South India.

DISCUSSION.

These surveys, while less extensive in respect of duration and numbers included than other surveys already carried out or in progress, are nevertheless worth

recording since they add something to our knowledge of dietary habits in India. It is of particular interest to note that the Travancore children were shorter and lighter than children living elsewhere in South India. The low average height and weight of the Travancore girls is particularly striking. There is no reason to suppose that this stunting of growth is due to racial difference. It can reasonably be ascribed to dietary factors, the most important of which is a low intake of protein. When tapioca or some other root forms a considerable proportion of the diet, protein intake is likely to be small. Experiments have been carried out in which groups of rats have been fed on diets largely composed of tapioca. Growth on such diets is very poor, being in fact poorer than that observed when diets based on rice, resembling those consumed by the poor in South India, are given. The addition to a tapioca diet of pure protein (casein) in quantities roughly equivalent to 45 grammes in the case of human diets greatly enhances the nutritive value of that diet for rats. The results of these animal experiments will shortly be published. It would appear, from the dietary investigations recorded here and the related experiments on rats, that the present tendency in certain parts of South India for tapioca to replace rice as a staple food is undesirable from the standpoint of nutrition. Tapioca is a highly productive food crop, giving very large yields per acre. It is nevertheless, because of its deficiency in protein and probably in other food factors as well, unsuited to form the main ingredient in diets which are largely deficient in protective foods.

SUMMARY.

1. The diets of tea plantation labourers in the Nilgiris and villagers in Travancore have been studied. Both groups consumed an unsatisfactory diet. That of the Travancore group was particularly low in protein because of the large intake of tapioca.

2. Boys and girls in the tapioca eating district were found to be smaller and lighter than children in other parts of South India living on a rice staple. This stunting of growth is ascribed to low protein intake.

ACKNOWLEDGMENT.

I acknowledge gratefully the help of Miss Ouwerkerk and Mr. Chakko in connection with the Travancore Survey.

REFERENCE.

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A SURVEY OF DIET AND NUTRITION IN NAJAFGARH, DELHI PROVINCE

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THE present investigation forms part of the scheme initiated by the Nutrition Advisory Committee of the Indian Research Fund Association at its first meeting in June 1936, which has as its object the collection of data about the diet and state of nutrition of population groups in sample areas throughout India.

THE AREA STUDIED.

The district (thana) of Najafgarh is situated at the south-west corner of Delhi Province. Its principal town, Najafgarh, with a population of about 5,000, is 17 miles from Delhi City. The total population of the area, according to the 1931 census, was 42,736.

A Health Unit, financed by the Government of India and the Rocketteller Foundation, was started in the district in 1937. The presence of the unit, which facilitated contact with the people, was one of the reasons why the area was chosen for survey.

Climate.—The climate is typical of the Punjab, with extreme heat in summer when the thermometer may mount to 115°F., and a cold winter with the day temperature falling to 36°F. or thereabouts. The 'cold weather' lasts from November to February; April, May, and June are the dry 'hot weather' months; July, August, and September are the 'monsoon' months of rain and heat. The usual annual rainfall is from 20 inches to 25 inches.

Mortality and disease.—The recorded death rate for the last few years was about 35 per mille in Najafgarh and 25 per mille in the surrounding villages, infantile mortality, 250 per mille urban and 210 rural; birth rate, 60 per mille urban, 40 rural. These figures must be regarded with great reserve, owing to defects in registration. More exact data will be collected as the Health Unit's work develops. It may,

however, be taken for granted that the death, birth, and infantile mortality rates are all high.

Malaria is very prevalent and high spleen rates have been recorded in the villages. There is much low-lying land, frequently under water during the monsoon months, which provides excellent breeding places for mosquitoes. No regular survey of the incidence of tuberculosis has been carried out, but it appears to be not uncommon. Outbreaks of cholera and plague have occasionally been reported. Small-pox has hitherto been common, but may be expected to decrease owing to the widespread introduction of vaccination in 1937. Leprosy is rare. Stone in the bladder is reported to be common in the eastern part of the district.

ECONOMIC CONDITIONS, FOOD SUPPLY, AND DIET.

The great majority of the population is Hindu, there being few Mohammedans in the district. Nearly all the Hindu communities of North India are represented. Najafgarh town is the only important market and it is here that any surplus produce is sold and the villagers buy necessities such as clothes, salt, etc. Outside Najafgarh town the entire population is engaged in agriculture and lives on food produced in the district. There is no modern irrigation and in bad seasons there may be danger of scarcity or famine.

The diet changes to some extent with season, following the succession of harvests. The principal food crops, and the seasons of sowing and harvesting, are as follows:—

Crop.	Time of sowing.	Time of harvesting.
Cambu (millet) (<i>Pennisetum typhoides</i>)	June	October.
Cholam (<i>Sorghum vulgare</i>) ..	June	October.
Black gram (<i>Phaseolus mungo</i>)	June to July	October.
Green gram (<i>Phaseolus radiatus</i>)		
Horse gram (<i>Dolichos biflorus</i>)		
Wheat	October	April to May.
Barley	October	March to April.
Bengal gram (<i>Cicer arietinum</i>) ..	September	March to April.

During the winter months millet and wheat are the principal cereals eaten. In the summer months wheat or barley are prominent in the diet : at this season mixtures of cereals and pulse, e.g., wheat or Bengal gram ; barley and Bengal gram ; or wheat, barley and Bengal gram, are commonly consumed.

Vegetables are grown in only a few villages. The chief reason for this is that water in most of the wells is brackish or salt ; in some villages there is only one sweet

water well for drinking purposes. Vegetable sellers from neighbouring villages may come once or twice a month to villages in which no vegetables are grown. The following vegetables are grown in the district : Spinach, fenugreek leaves, mint leaves, onions, brinjals (*Solanum melongena*), lady's fingers (*Hibiscus esculentus*), cucumber, bitter gourd (*Momordica charantia*), rape leaves. Bengal gram leaves, carrots, carrot leaves, bathua leaves. In general, intake of vegetables is very small.

Fruits.—There are hardly any fruit gardens in the district and the population cannot afford to buy imported fruits. Intake of fruit is almost negligible.

Milk and milk products.—These are derived from both cows and buffaloes, the latter being the most important milking animal. The average daily yield per buffalo is about 11 pounds as compared to about 4.5 pounds per cow. In the villages every household possesses one or two milch buffaloes.

During the buffalo breeding season—July to September—milk is plentiful and can be bought in Najafgarh town at 30 lb. to 35 lb. per rupee. During the dry season—April to June—milk is scarce and its cost rises to 10 lb. to 15 lb. per rupee. At this season one may find that in a village of 26 to 30 families only 2 or 3 families can obtain milk.

The practice of selling milk is increasing daily, this being the result of proximity to Delhi City and Delhi Cantonment. It is probable, however, that milk intake is higher in the Najafgarh area than in villages nearer the Delhi market. Another factor which makes for a reduced milk consumption is the export of good milch cattle to Bombay and Calcutta.

Ghee (clarified butter) is consumed by those who can afford it. Mustard oil is the most important vegetable fat.

Meat and eggs.—Tradition and taboo prevent the eating of these foods. The great majority of the population is Hindu and does not take meat at all, while the few Mohammedans take meat very rarely. The game present in the district is not killed for food. One community—the chamars (shoe-makers)—consumes animal carcasses.

Sugar.—Little sugar-cane is grown in the area. Some gur (unrefined sugar) is bought.

The preparation and cooking of food.—The whole cereal is ground into flour and is usually made into chapatties (flat unleavened cakes heated on iron pans). These may be made either of wheat or of mixtures of wheat, barley and Bengal gram. Cambu and cholam are cooked singly and consumed in the form of chapatties. Mixtures of cereals and pulses may be boiled and consumed as porridges. To such mixtures butter-milk may be added and the whole kept for some hours to allow fermentation to occur. Leafy vegetables are boiled and mashed, all preparations made from green leaves being called 'sag'. Milk, which is always boiled, is consumed in various ways. It may be taken as whole milk, as whole milk curdled (curds), or as an ingredient in sweet dishes and various other food mixtures. A common practice is to remove fat by churning curdled milk and drink the butter-milk which remains. This is usually diluted with varying amounts of water. Watery solutions of butter-milk are taken at any time of the day, being particularly relished in the hot weather.

Conditions of labour.—The villagers spend their time either at work in the fields or at home doing nothing. In the hot weather they begin work in the fields at 3 to 4 a.m., and carry on until 10 to 11 a.m. In winter work continues all the day. The women help the men in the fields or at other forms of manual labour, and in addition have other work such as grinding flour, drawing water, milking and feeding cattle, curdling and churning milk, and preparing meals. Actually the women do more manual work than most of the men. All except very young children assist in the labour of the family.

DIET SURVEYS.

In order to obtain a more accurate knowledge of food habits a number of quantitative diet surveys were carried out in various parts of the district.

The usual methods employed in the diet surveys of the Coonoor Laboratories were followed. House-to-house visits were made morning and evening to weigh uncooked food about to be consumed. In most cases the survey lasted 10 days. The recording of butter-milk consumption presented difficulty, since butter-milk may be diluted to any extent. To overcome this difficulty a lactometer was used, rough experiments have previously been carried out to correlate lactometer readings and the degree of dilution. By this means a sufficiently accurate estimate was obtained. In Table II the quantities of butter-milk consumed are stated in terms of the original whole milk.

For calculating intake of calories, etc., the tables given in Health Bulletin No. 23 (1937) were used. The usual League of Nations (1932) scale of family consumption co-efficients was employed.

In all, 101 families, comprising 531 persons, were studied. In addition the diet of the District Board School Hostel, housing 19 persons, was investigated. Families may be grouped as follows :—

- I. Twenty-five families engaged in very hard work, i.e., road-making.
- II. Ten families of townspeople living in Najafgarh town.
- III. Sixty-six families living in four different villages. Of these 44 were engaged entirely in agriculture, while the remainder followed occupations ancillary to agriculture (pot-makers, carpenters, etc.). The inquiries in the various villages were carried out at different seasons, and the results of these inquiries are grouped separately in Tables I (group IIIa, b, c, and d) and II.
- IV. Nineteen persons living in the District Board School Hostel. An investigation was carried out in the hostel in February and again in May, with the object of comparing food consumption in cool and hot seasons.

ECONOMIC STATUS.

Group I consisted of coolies earning from 4 to 6 annas per day. All family members, except young children, joined in the work and were wage-earners.

Group II consisted of small tradesmen with monthly incomes ranging from Rs. 8 to Rs. 30.

Group III was chiefly composed of families living on the produce of their own land. Particulars of the amount of land owned, annual yields, and their value were collected, but since these seem somewhat outside the scope of the present article, they are not included. The villagers keep sufficient grain for their own consumption and sell any surplus. Most of the families owned some livestock including cows and she-buffaloes.

Group IV consisted of boys between 11 and 15, a teacher, and two kitchen servants. The boys came from neighbouring villages. It was their practice to go to their villages every Sunday and obtain wheat, flour, ghee, etc. The wheat and flour were collected in a store from which they could be issued morning and evening.

THE NUTRITIVE VALUE AND COMPOSITION OF DIETS.

Table I gives the average intake of calories, proximate principles, calcium, and phosphorus in the various groups. The period covered by each investigation is shown in column 3. Mean intake of the principal foodstuffs is shown in Table II.

Mean calorie intake, as Table I shows, was above 2,600 per day in all groups and may be regarded as satisfactory. The mean for group III composed of village families (3,080) may be compared with that calculated for 29 village families in South India living on a rice staple (2,400) by Aykroyd and Krishnan (1937). Protein intake in all the Najafgarh groups was much higher than in the South Indian group; in the latter it was only 63 grammes daily. The advantage of the North Indian population in this respect is due to the consumption of a wheat instead of rice staple and a higher intake of pulses and milk. Some animal protein, derived from milk, was included in the diet, while intake of animal protein in the South Indian group was negligible. The calcium content of the Najafgarh diet was fairly high because of the large intake of whole wheat and the not inconsiderable milk consumption; mean intake of calcium compares well with that of the South Indian families referred to, which was 0.31.

Intake of vitamin A and carotene was fairly high in most groups, the former being obtained from milk fat and the latter from whole wheat and Bengal gram. All the diets contained more than 1,000 International units of vitamin B₁, derived from whole cereals, and were thus more than adequate in this respect. With regard to vitamin C, however, intake was low owing to the low consumption of vegetables and the absence of fruits in the village group. It was calculated to be about 4 mg. per consumption unit per day, which is far below current estimates of human requirements. It must, however, be remembered that the surveys covered only short periods and that at other seasons a little more fruit and vegetable may be obtainable.

Table II shows that whole wheat was the main item in diet of all groups except group IIIa, in which millet (cambu) was the most important cereal. Intake of milk and milk products was reasonably high in certain groups; group I, however, consumed no milk. The fact that milk intake in villages *c* and *d* of group III was lower than that in villages *a* and *b* may be ascribed to the fact that the surveys in the latter was carried out later in the year; as the hot weather advances, the cows and she-buffaloes go dry. It is clear, however, that milk consumption in the villages in this district is superior to that observed in Aykroyd and Krishnan's investigation in South India, which was repeated

TABLE I.

Intake of calories, proximate principles, calcium, and phosphorus per consumption unit per day.

Group.	Locality.	Period of inquiry.	Number of families.	Number of persons.	PROTEIN.			FAT.			Carbohydrates.	CALORIES.		Calcium.	Phosphorus.
					Total.	Animal.	Percentage of animal protein.	Total.	Animal.	Percentage of animal fat.		Total.	Percentage derived from cereals.		
I ..	Cooly lines, Najafgarh.	23-12-37 to 29-12-37	25	85	141.3	27.4	782.2	3,940	71.5	1.21	3.28
II ..	Najafgarh town	21-1-38 to 2-2-38	10	45	100.6	7.3	7.2	39.3	15.8	40.3	556.8	2,900	68.6	1.01	2.39
III (a)	Chawlah village	15-2-38 to 24-2-38	14	82	110.0	11.9	10.6	64.6	32.7	50.6	538.2	3,220	73.8	1.15	2.92
" (b)	Surera village	25-3-38 to 3-4-38	9	69	119.5	14.0	11.7	56.1	30.8	54.8	599.2	3,370	68.4	1.14	2.79
" (c)	Asalpur village	3-5-38 to 12-5-38	26	132	92.0	4.9	5.3	36.9	16.2	44.0	481.7	2,620	64.8	0.76	2.04
" (d)	Sarakhpur village.	5-6-38 to 12-6-38	17	118	110.3	3.4	3.0	19.8	3.7	18.5	620.4	3,100	86.3	0.55	2.45
IV ..	School hostel, Najafgarh.	(a) 7-2-38 to 22-2-38	..	19	86.3	4.6	5.3	75.0	65.7	87.6	479.7	2,970	67.0	0.71	2.14
		(b) 25-5-38 to 11-6-38	..	17	78.9	2.7	3.4	72.5	63.8	87.9	413.9	2,630	68.0	0.52	1.95

TABLE II.

Principal foodstuffs in the diet of the various families. (Oz. per consumption unit per day.)

Foodstuff.		Group I.	Group II.	GROUP III.				GROUP IV.	
				a	b	c	d	a	b
Cereals	Whole wheat ..	26.5	10.9	2.5	10.7	14.0	8.9	20.0	18.2
	Cambu ..	2.1	5.8	18.2	7.9	..	1.3
	Barley	2.8	2.5	4.4	2.8	17.7
	Pulses (chiefly Bengal gram).	8.4	4.2	2.6	5.4	5.6	2.2	1.9	1.9
Vegetables	Leafy ..	2.4	2.0	1.5	1.8	0.3	..
	Root and tubers	0.5	2.9	0.5	0.8	0.1	..	5.0	..
	Other vegetables	..	0.1	..	0.3	0.2	0.3	0.7	6.9
Milk and milk products.	Milk	5.5	10.8	10.4	4.2	3.57	3.3	2.0
	Butter-milk	0.2	3.1	4.0	4.7
	Ghee	0.3	0.5	0.8	0.4	..	2.5	2.9
Sugar or jaggery ..		1.5	1.5	0.4	0.7	0.8	..	0.33	..

No meat or fruit was consumed during the periods of inquiry.

at two seasons ; about 75 per cent of the South Indian families consumed no milk at all, while consumption in the remainder rarely exceeded 5 oz. per consumption unit per day. Of the 66 families in group III, 17 (25.7 per cent) consumed no milk during the period of investigation. Other salient points about the diets are the high pulse intake and the complete absence of meat and fruit.

Attention may be drawn to one point of interest arising out of the survey of the small school hostel group (IV). The ages of the boys ranged from 11 to 15. The first survey (IVa) was made in February, when the weather is still cool, the second in the hot weather in May, when the thermometer may rise to 115°F. In the latter month calorie intake per consumption unit was estimated to be 2,630 as compared with 2,970 in February. This suggests that while calorie needs in the hot weather may be reduced, the saving is not very great. The surveys in the hostels and the villages do not support the view which is sometimes expressed that in the hot weather in India 'much less food is required than in the cold weather'.

THE STATE OF NUTRITION OF SCHOOL CHILDREN.

A survey of school children was carried out on the lines of the survey of Aykroyd and Rajagopal (1936) in South India. There are 28 schools in the district, situated

in different villages. The various schools were visited in turn and all the children present examined. Education is not compulsory and many children do not attend school at all. The investigation included 1,483 children, 1,339 boys and 144 girls, all that could be found in the schools for examination in a district with a population of over 40,000. In general the children examined belonged to the families of the type included in the survey, some being members of these families.

The children were weighed and measured. For weighing a spring machine was used, the accuracy of which was tested regularly by putting known weights on it. Weights were recorded to the nearest pound and heights to the nearest quarter of an inch. The children were weighed in shirts with dhoties or pyjamas and without shoes, caps, or turbans. Date of birth was taken from school registers; the accuracy of the registers, however, cannot be guaranteed, because many families do not keep any record of the date of their children's births.

The children were also examined for the presence of angular stomatitis, xerophthalmia, phrynoderma, and caries and malocclusion of the teeth.

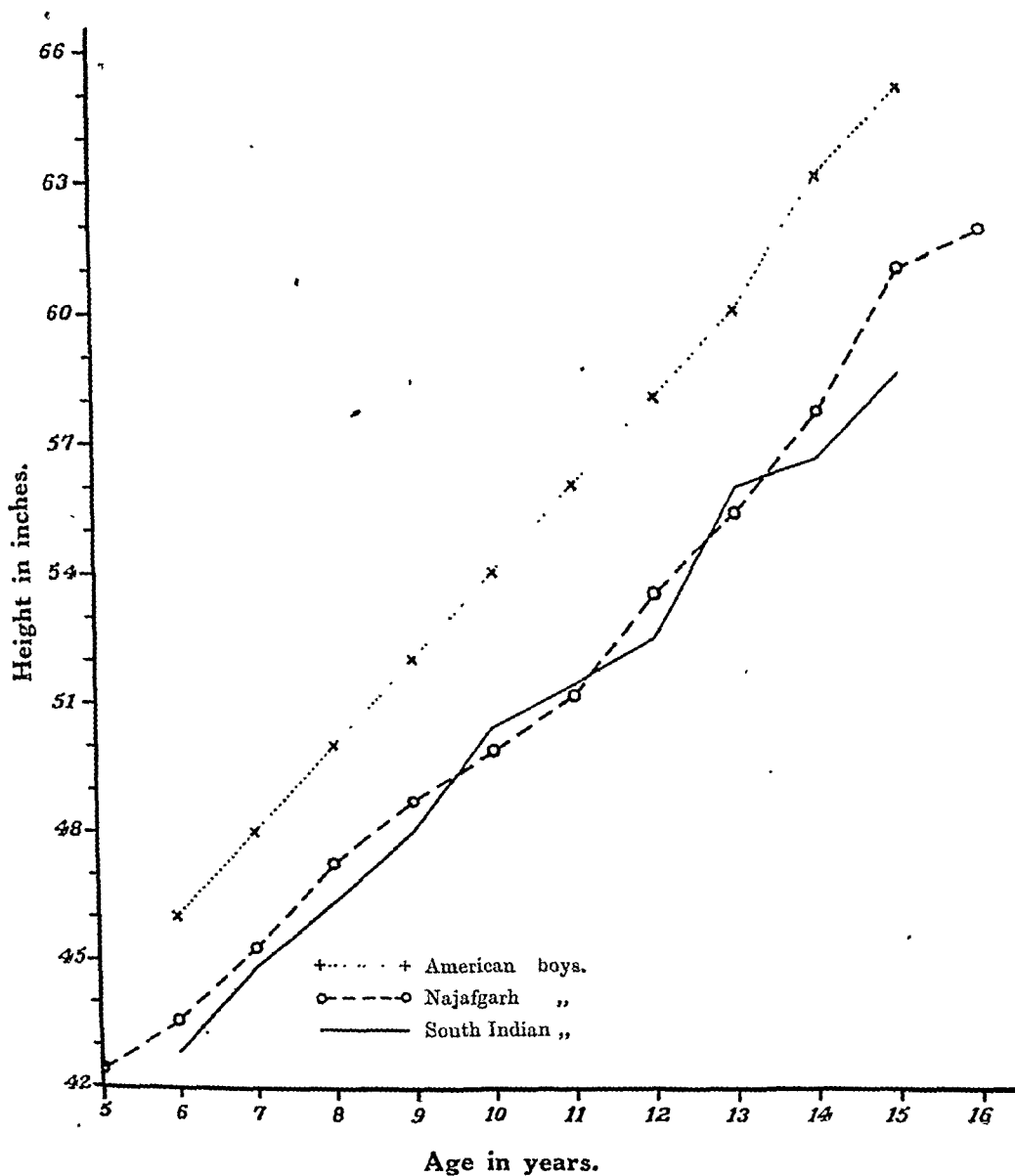
Table III shows the average weight and height of boys in the age groups 5 to 17. Averages for South Indian boys are shown for purposes of comparison, the latter figures being based on data collected by the Coonoor Laboratories. In Charts 1 and 2 these figures are shown graphically, together with averages for better class American boys (Baldwin, 1924).

TABLE III.

Average weight and height for age of boys in Najafgarh district and of South Indian boys.

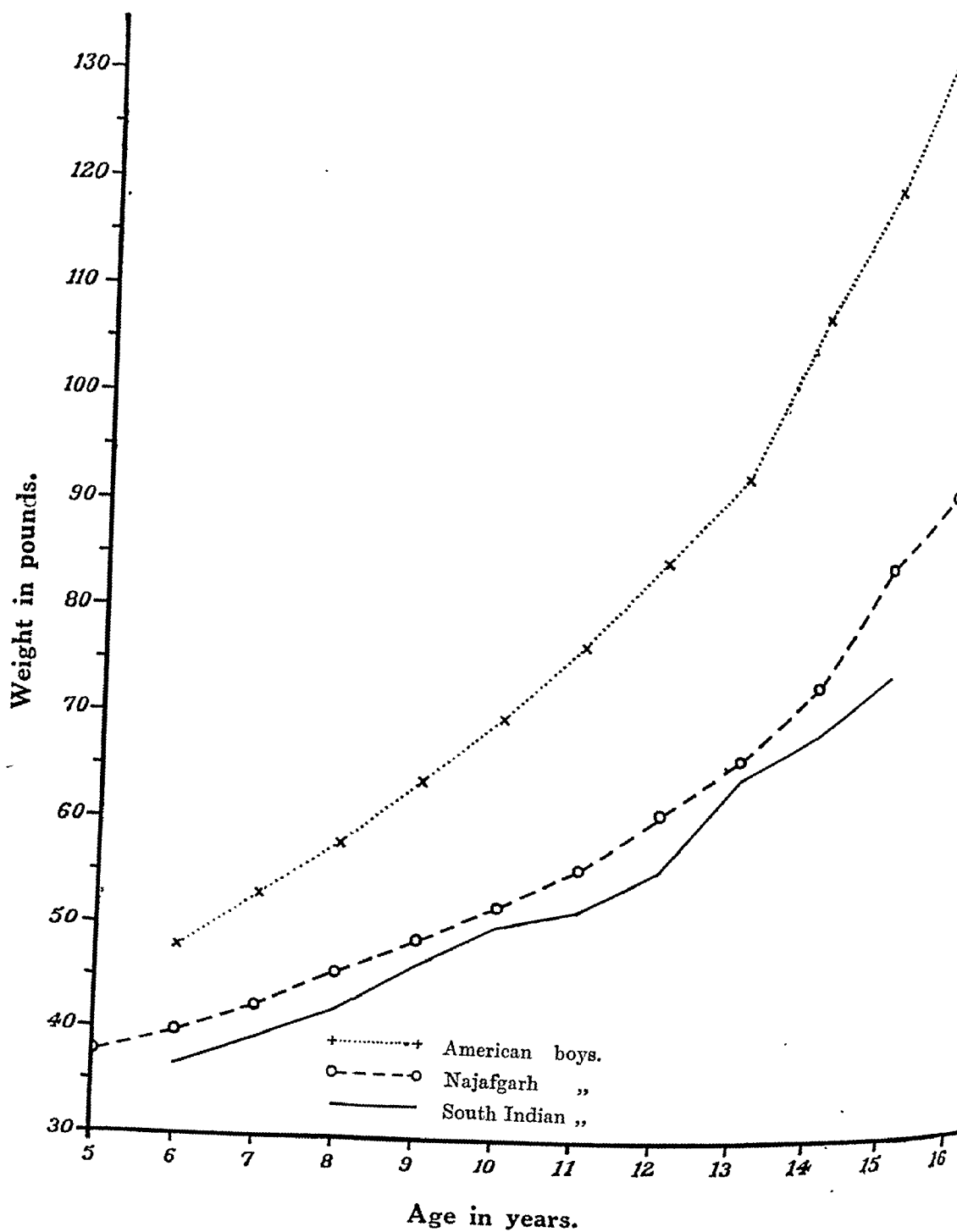
Age.	NAJAFGARH AREA.			SOUTH INDIAN BOYS.		
	Number weighed and measured.	Height in inches.	Weight in lb.	Number weighed and measured.	Height in inches.	Weight in lb.
5	14	42.5	37.8
6	72	43.6	39.9	122	42.8	36.6
7	142	45.3	42.2	184	44.8	39.2
8	170	47.3	45.8	186	46.4	42.1
9	183	48.7	48.8	177	48.0	46.5
10	164	49.9	52.0	203	50.4	50.2
11	135	51.2	55.8	168	51.4	51.6
12	119	53.5	61.1	133	52.5	55.6
13	106	55.4	66.6	107	55.9	64.5
14	108	57.7	73.6	53	56.6	68.7
15	73	60.9	84.5	49	58.5	74.1
16	38	61.8	91.0
17	15	64.4	105.1

CHART 1.



Height-age averages in Najafgarh, South Indian, and American boys.

CHART 2.



Weight-age averages in Najafgarh, South Indian, and American boys.

It is to be observed that the height averages of the Najafgarh and South Indian boys are closely parallel. On the other hand, the Najafgarh boys are heavier (3 lb. to 5 lb.) at all ages—a fact which can also be expressed by saying that in this group the weight attained for a given height is superior.

The incidence of clinical signs of deficiency disease.—It has been shown, by workers attached to the Coonoor Laboratories and others, that a valuable and practicable method of determining the state of nutrition of a population is the observation of the incidence of certain clinical signs of deficiency disease. These are phrynoderma, angular stomatitis, and xerophthalmia. Reference has been made to these conditions, and their use in the assessment of state of nutrition, in a series of papers from the Coonoor Laboratories.

In the present investigation the children were examined for the presence of these signs. The results are shown in Table IV:—

TABLE IV.

Incidence of clinical signs of deficiency disease.

	Number examined.	Number showing clinical signs.	Per cent- age.	Number showing phryno- derma.	Per cent- age.	Number showing angular stomatitis.	Per cent- age.	Number showing xeroph- thalmia.	Per cent- age.
Boys ..	1,339	55	4.1	17	1.3	15	1.1	23	1.7
Girls ..	144	4	2.8	2	1.4	3	2.1

The percentage of children suffering from signs of deficiency disease was in general lower than that observed in South Indian investigations (Aykroyd and Rajagopal, *loc. cit.*; Aykroyd and Krishnan, *loc. cit.*). The incidence of xerophthalmia was less than in Assam children as recorded by Wilson and Mitra (1938). Severe cases of angular stomatitis were not observed. The general condition of the children examined was fairly good, being visibly superior to that of poorer class South Indian children.

Teeth.—The teeth of all the children whose measurements were recorded were examined for dental caries and malocclusion. In recording the results of the caries investigation the children were divided into three groups: (a) 6 to 8, (b) 9 to 11, and (c) 12 to 15.

Table V shows the incidence of gross caries in various age groups (boys). It is to be observed that the examination for caries was simply an

inspection of the mouth to note obvious decay. Probe and mirror were not employed.

TABLE V.

Incidence of gross caries.

Age.	Number of boys examined.	Percentage incidence of gross caries.
Boys between 6 and 8 (inclusive) ..	384	10.0
Boys between 9 and 11 (inclusive) ..	482	9.5
Boys between 12 and 15 (inclusive) ..	406	4.7

Dental decay was observed in 10.0 per cent of the boys between the ages of 6 and 8 and in 9.5 per cent of the group 9 to 11. In boys above the age of 12 the incidence was only 4.7 per cent. Considerable importance must be attached to this observation since it suggests that dental caries is increasing in the district as the drain of milk to the city grows. In inaccessible villages at a distance from a big town the amount of caries among children is very low.

In European children, according to data collected by Mellanby (1934), the percentage showing some degree of caries is in the neighbourhood of 90 per cent. The relative infrequency of caries in the Najafgarh children is thus an observation of considerable interest, though it must, of course, be remembered that only gross caries would be detected by the method of investigation followed.

Malocclusion.—Table VI shows the incidence of certain types of dental irregularity to which the term malocclusion has been applied. Only children above 9 years of age.

The general result of this investigation was to show that the majority of children were not only free from gross caries but had also well-formed regular teeth and did not show the irregularities in position which are so common in certain parts of the world. Radhakrishna Rao (unpublished observations) has observed that in South India some 40 to 60 per cent of urban school children show irregularities and displacements of the teeth in the lower jaw suggestive of over-crowding. It may be suspected that some lack of development of the jaws, possibly due to diet deficiency, is responsible for the high incidence of 'malocclusion' in South India.

TABLE VI.
Malocclusion.

AGE:—	9	10	11	12	13	14	15
	183	164	135	119	106	108	73
NUMBER OF BOYS:—							
Percentages.							
<i>Upper jaw.</i>							
(a) Teeth crowded with overlapping of crowns.
(b) Teeth rotated about axis	0·7	0·8	1·8
(c) Large gaps between incisors (central or lateral).	24·5	15·8	8·5	4·2	8·4	2·7	4·1
(d) Incisor teeth protruding in the shape of fan.	1·6	1·8	1·4	3·3	4·7	2·7	..
(e) Lateral incisors erupted behind central incisors.
(f) Other irregularities
<i>Lower jaw.</i>							
(g) Teeth crowded with overlapping of crowns.	2·1	2·4	0·7	..	1·8	0·8	1·3
(h) Rotation about axis ..	9·8	7·9	3·7	1·7	4·7	2·7	1·3
(i) Lateral incisors erupted behind central incisors.	3·2	4·2	3·7	..	0·8	4·6	1·3
(j) Other irregularities ..	0·5

DISCUSSION.

The diet of the population studied in the present investigation may be described as moderately satisfactory. The fact that it is largely based on whole wheat and contains milk in fair quantities makes it definitely superior in many respects to diets consumed in the rice-eating areas of India. The superiority of whole wheat to rice as a staple cereal is particularly marked when rice is consumed in a highly milled state.

On the other hand, the Najafgarh diets are very deficient in vegetables and fruit and this appears to be their most important defect. Intake of vitamin C is low. It is to be observed that, while the general condition of the children is by no means unsatisfactory, infantile and probably maternal mortality in the area are high. To what extent dietary factors play a part in this it is impossible to say.

To remedy the shortage of vegetables, the encouragement of kitchen gardens is desirable. These could be situated in one corner of the large yard surrounded by mud walls in which cattle are kept, and irrigated by waste water from the house. A small plot can grow a surprising quantity of vegetables.

The theory that dental caries is related to deficiency of vitamin C receives no support from the present investigation. Intake of vitamin C is undoubtedly low, yet the children examined are remarkably free from gross caries as compared with children in Europe and the United States.

SUMMARY.

1. A survey of diet and nutrition has been carried out in Najafgarh, Delhi Province. The diet of 101 families, comprising of 531 persons, was studied for a period of about 10 days. In addition the diet of the District Board School Hostel, housing 19 persons, was investigated during the cool and hot seasons.

2. The diets, based on whole cereals and containing fair quantities of milk, were found to be satisfactory in certain respects. There was, however, a marked deficiency of vegetables and fruit, which represents the chief defect of the diet.

3. Calorie intake in the school hostel was found to be somewhat reduced in the hot weather, but the reduction was not very great.

4. The 'state of nutrition' of 1,483 children was investigated. The average height of the boys was similar to that of South Indian boys, but the Najafgarh boys were a little heavier at all ages. Signs of deficiency disease were rare and the general condition of the children was good.

5. Ninety to ninety-five per cent of the children were free from gross caries and dental irregularities.

The detailed records of the surveys are being preserved in the Nutrition Research Laboratories, Coonoor, and can be made available to any one wishing to use them for economic investigations, etc.

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NUTRITIONAL INVESTIGATIONS ON BENGAL FISH.

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FISH constitutes one of the most important ingredients of the daily diet in Bengal among all classes of the population. The reason, no doubt, is that the Bengal delta is covered with a network of rivers and canals and a considerable supply of fish is available more or less throughout the year. The number of varieties of fish that are eaten is very large, though a few of them are particularly popular.

The nutritive values of these fish obviously deserve systematic investigation and we commenced work on this subject in 1932. Our previous investigations were concerned mostly with vitamin-A, -B₁, and -B₂ values of a number of Bengal fresh-water fish and we found that the liver oils of some of these are considerably more potent in vitamin A than cod-liver oil, though not as rich as halibut-liver oil, and the body fats are, of course, very much poorer than the liver oils (Chakravorty, Mookerji and Guha, 1933 ; Ghosh, Chakravorty and Guha, 1933 ; Ghosh and Guha, 1934, 1935.) We considered it desirable to extend this research to include vitamin, protein, fat, and mineral contents of the different varieties of fish. It is known that the chemical composition of the same species of fish varies considerably with reference to season as well as the reproductive cycle and we intend to investigate such varieties in future. The effect of different methods of freezing and cold storage on the composition of fish seems to us to be worthy of study, as in Bengal owing to seasonal gluts a very large quantity of fish is allowed to perish every year. Valuable work in this connection has been done with reference to the fish of British waters by the Food Investigation Board of Great Britain (*vide* their Report for 1936). It seems also worth while to investigate whether some sea-fish is available in the Bay of Bengal which might supply a liver oil as a steady source of vitamins A and D, for although the liver oils of some fresh-water fish

are potent sources of vitamin A, they cannot afford a large and steady supply throughout the year.

The present communication gives the results concerning the total water, body fat, total mineral matter, protein, ionizable iron, total iron, total calcium, and total phosphorus of 24 different varieties of Bengal fresh-water fish, and further results will be published in subsequent communications.

EXPERIMENTAL.

Method of sampling.—As a rule, after recording the total weight of the fish and its general condition, the edible portion is separated from the bones and other non-edible portions. Ordinarily the fleshy portion with its small proportion of inseparable tissue is analysed. The large-sized piece obtained by separating is first cut into slices and these are then run through an 'Enterprise' hasher twice for uniform mixing.

Determination of moisture.—Fifty grammes of finely hashed sample are weighed by difference in a small porcelain dish and dried in a low temperature drying oven, the temperature maintained being 40°C. Drying, cooling, and weighing are continued until the weight is constant.

Determination of ash or total mineral matter.—Ten grammes of the dried sample are ignited in a silica dish. The charred mass is extracted with hot water, filtered through an ashless filter-paper, and the residue again ignited to a white ash. The extract obtained with the hot water digestion is evaporated to dryness in the same dish.

Estimation of fat.—Ten grammes of the dried sample obtained after the determination of moistures are finely powdered, taken in an extraction thimble, and extracted with petroleum ether for 16 hours in a Soxhlet. The solvent is evaporated from the extract, taken in a small glass basin on an electric-bath. The last traces of the solvent are removed in an air-oven at 45°C. The residue is cooled in a desiccator and weighed.

Determination of protein.—Total protein is estimated by the usual Kjeldahl method. The nitrogen value was multiplied by the factor 6.25.

Determination of ionizable iron in the raw fish.—The method adopted was that of Hill (1930). The raw hashed fish sample is again thoroughly pulped in a mortar. Five grammes of the sample are weighed and taken in a test-tube of 40 c.c. capacity and heated at 100°C. for 10 minutes. To this 10 c.c. of sodium acetate and acetic acid buffer at pH 5.5 were added. After testing the pH of the fluid in the tube, 0.5 g. to 1 g. of sodium hydrosulphite (free from iron) is added. The content of the tube is mixed well and allowed to stand over-night. The volume is then made up to 20 c.c. with distilled water and filtered through Whatman filter-paper and again centrifuged. To the clear solution thus obtained a small crystal of α - α -dipyridyl is added and mixed well. The colour thus developed is matched against the standard and the amount of iron determined.

For the estimation of iron, calcium, and phosphorus, the ash obtained by the method described was taken up in dilute hydrochloric acid and any insoluble residue was filtered off. The filtrate was made up to a known volume.

Aliquots of this solution were used for the determination of iron, calcium, and phosphorus.

Estimation of iron.—Iron determination was carried out by the method of Elvehjem (1930). Ten c.c. of the above solution were taken, made distinctly alkaline with 40 per cent sodium hydroxide (iron-free), and boiled for an hour. This was cooled, made acid with dilute hydrochloric acid, and diluted to a volume of 50 c.c. The colorimetric estimation was then carried out as outlined by Kennedy (1927). One c.c. of the standard iron solution equivalent to 0.1 mg. of iron was mixed with 5 c.c. dilute hydrochloric acid and diluted to 50 c.c. Aliquot portions (10 c.c.) of the standard and of the unknown were placed in stoppered cylinders, 10 c.c. of amyl alcohol and 5 c.c. of 20 per cent potassium thiocyanate were added in each cylinder and the mixture was thoroughly shaken. The coloured layers of amyl alcohol were then compared in a colorimeter.

Estimation of calcium.—A known volume of the ash solution was made just alkaline with ammonia, excess of 20 per cent ammonium acetate (10 c.c. to 15 c.c.) was added to it and the solution was freed from phosphates by means of dilute ferric chloride. This was filtered and washed with 1 per cent ammonium acetate. The filtrate and washings were boiled with 2 g. to 3 g. ammonium chloride. One to 2 g. of oxalic acid crystals were added to the boiling solution with continual stirring, a few drops of strong ammonia and a sufficient quantity of 3.5 per cent ammonium oxalate solution were added while the stirring was continued. The precipitate of calcium oxalate was filtered, washed free from chloride with 1 per cent ammonium oxalate solution, and burnt to calcium oxide in a silica crucible. The above method was adopted from that of Richards, McCaffrey and Bisbee (1901). The calcium was calculated as calcium oxide.

Estimation of phosphorus.—The method adopted was that of Burns and Henderson (1935). An aliquot portion of the ash solution was taken; 5 c.c. to 10 c.c. of concentrated nitric acid were added to it, which was followed by 20 c.c. to 30 c.c. of 34 per cent ammonium nitrate solution, and finally 70 c.c. to 80 c.c. of 3 per cent ammonium molybdate solution were added. The solution was heated and the precipitate of ammonium phosphomolybdate allowed to settle. The precipitate was filtered and freed from acid by washing with hot water. The precipitate was then dissolved in a known volume of 0.5 N sodium hydroxide solution and the excess of alkali titrated with 0.5 N hydrochloric acid, phenolphthalein serving as indicator. One c.c. of 0.5 N sodium hydroxide was equivalent to 0.675 mg. of phosphorus.

Tables I and II give the results concerning the mean values of water, body fat, ash, protein, ionizable iron, total calcium, total iron, and total phosphorus of the different varieties of fish, obtained usually from analyses of 4 to 7 samples of each kind. The zoological names of a few of these have not been obtained. Estimations of the glycogen and carbohydrates of the fleshy portion indicated that they are usually present in negligibly small amounts. In the analysis of the specimen there is usually a deficit of 3 to 6 per cent, which has not been accounted for and perhaps relates to extractives, etc. The percentage in Table I is given on the basis of fresh raw fish and the ranges of weights (column 4 of the table) indicate weights of the maximum and minimum sizes then available in the market and analysed.

	Tengra	..	25-70	70	6·4	2·1	19·2	97·7	0·31	Fleshy edible portion.
22-7-38	Bhangar bata	..	111-214	67·3	4·4	2·2	19·4	93·3	0·24	Do.
25-7-38	Singhi	..	21-69	68	0·6	1·7	22·8	93·1	0·97	Do.
29-7-38	Mugil parsia	..	8-22	69·3	5·9	2·1	16·6	93·9	0·51	Fleshy edible portion and one species with bones.
26-7-38	Glassgobius giuris	..	39-405	70·7	0·6	2·3	14·5	97·1	0·63	Fleshy edible portion.
28-7-38	Ophicephalus punctatus	..	32-55	74	0·6	2·6	10·4	96·6	0·72	Do.
29-7-38	Anabas testudineus	..	31-74	70	8·8	2·0	14·8	95·6	0·74	Do.
30-7-38	Notopterus notopterus	..	108-270	73	1·0	2·5	19·8	96·3	0·51	Do.
3-8-38	203-1,121	70·6	8·8	2·0	14·8	96·2	0·71	Do.
14-8-38	Labeo rohita	..	160-12,600	76·7	1·4	0·9	16·6	95·6	0·50	Do.
14-8-38	Khoyra	..	24-39	72	3·0	1·8	18·0	94·8	0·23	Do.
24-4-38	Catla catla	..	620-22,500	73·7	2·4	1·5	19·5	97·1	0·55	Do.
5-4-38	Wallago attu	..	730-1,200	73	2·7	1·3	15·4	92·4	0·36	Do.
20-4-38	Labeo calbasu	..	316-835	81	1·0	1·3	14·7	98·0	0·26	Do.
7-4-38	Lates calicifer	..	250-1,215	82	1·1	1·2	13·7	98·0	0·35	Do.
11-4-38	Clarius batrachus	..	88-214	78·5	1·0	1·3	15·0	95·8	0·75	Do.

TABLE II.

Mean values of total calcium, total iron, and total phosphorus in fish.

Bengali name.	Zoological name.	Calcium in g. per 100 g. of raw fish.	Iron in mg. per 100 g. of raw fish.	Phosphorus in g. per 100 g. of raw fish.
Bele ..	<i>Glassgobius giuris</i>	0.37	104	0.33
Lata ..	<i>Ophicephalus punctatus</i>	0.61	130	0.53
Kôï ..	<i>Anabas testudineus</i>	0.41	135	0.39
Fôlui ..	<i>Notopterus notopterus</i>	0.59	169	0.45
Bhangar	0.182	121	0.19
Rohu ..	<i>Labeo rohita</i>	0.68	85	0.15
Khoyra	0.59	67	0.22
Katla ..	<i>Catla catla</i>	0.51	76	0.21
Boal ..	<i>Wallago attu</i>	0.16	62	0.49
Kalabasu ..	<i>Labeo calbasu</i>	0.32	83	0.38
Bhetka ..	<i>Lates calcifer</i>	0.53	102	0.40
Magur ..	<i>Clarius batrachus</i>	0.21	74	0.29
Fesha	0.44	115	0.34
Mrigal ..	<i>Cirrhina mrigala</i>	0.35	109	0.28
Sole ..	<i>Ophcephalus striatus</i>	0.14	54	0.095
Kuja vetki	0.46	78	0.23
Mowrala ..	<i>Amblypharynx godonmola</i>	0.55	90	0.35
Puti	0.11	96	0.096
Hilsa ..	<i>Clupea ilisa</i>	0.18	213	0.28
Bugda chinghri	0.29	137	0.42
Tengra	0.27	201	0.17
Bhangar bata	0.58	112	0.31
Singhi ..	<i>Saccobranchus fossilis</i>	0.67	226	0.65
Parsey ..	<i>Mugil parsia</i>	1.05	205	0.70

SUMMARY.

The percentage quantities of water, body fat, ash, protein, and available iron in 24 different varieties of Bengal fresh-water fish are given. The well-known fatty

fish, hilsa (*Clupea ilisa*), has the highest fat content, 19·4 per cent, although its liver oil, as we had shown in earlier publications (*loc. cit.*), is not as potent in vitamin A as that of some other fish like rohu (*Labeo rohita*) whose total fat content is very much lower. The next best sources of fat are kôl (*Anabas testudineus*) and bhangar, each of which gave a figure of 8·8. The protein content, 22·8 per cent, is highest in singhi (*Saccobranchus fossilis*), which is also the best source of available iron. This fish has at the same time the lowest content of fat, 0·6 per cent. These observations may be related to the fact that this fish is popularly considered to be a valuable item of convalescent diet in Bengal. The calcium content, 1·05 per cent, is highest in parsey (*Mugil parsia*), which is also the richest source of phosphorus, viz., 0·7 per cent, among the varieties analysed. The next good sources of calcium are singhi (*Saccobranchus fossilis*) and rohu. As regards the total iron content, singhi (*Saccobranchus fossilis*) headed the list, viz., 226 mg. per 100 g. of the raw fresh fish; then come parsey and tengra.

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ON COPPER CONTENT OF FOODS.

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RECENT researches on experimental anæmias have revealed the importance of copper as a factor in the formation of hæmoglobin of blood. Anæmia produced in young rats by a diet consisting mainly of cow's milk was found by Waddell *et al.* (1928) to be cured by iron plus copper and not by iron alone. Cunningham (1931) further pointed out that the iron was stored in the liver in the absence of copper and that, after the administration of copper, the stored iron was used for hæmoglobin formation. This relationship between iron and copper also finds support from the investigations of Josheps (1932) as well as those of Elvehjem and Sherman (1932).

The question now arises how far these experiments point to the necessity and importance of copper in human nutrition when in the above experimental anæmias the amount of copper required for the conversion of the absorbed iron into blood hæmoglobin is very small (Rose *et al.*, 1934). On the contrary, the metal being widely distributed in food materials should be amply supplied by an average mixed diet to furnish sufficient copper to stimulate hæmatopoietic action. The case, of course, might be different with children as copper like iron is present in very minute quantities in milk. Indeed results have already been published (Elvehjem *et al.*, 1935; Usher *et al.*, 1935; Goldstein, 1935) to show that small amounts of both iron and copper are often necessary to insure optimum hæmoglobin formation in the blood of infants. The occurrence of the metal in all animal and vegetable products further points to its necessity for animal as well as plant growth. Naturally, therefore, from the nutritional point of view it would be of interest to know the percentages of the metal present in the different foodstuffs usually consumed by people of any locality. Accordingly, certain foodstuffs available in the local market were analysed and the results of investigations are being recorded in this paper.

As the intention was merely to have a general idea on the relative amounts of copper present in different food materials, the determination of moisture though initially studied for certain substances (spinach containing 94 per cent; brinjal, 92.9 per cent; soya bean, 6.43 per cent; 'patôl', 88.9 per cent) was afterwards considered to be of no practical value, and the amount of copper was directly ascertained.

On Copper Content of Foods.

The vegetables, leafy and non-leafy, and fishes were first washed with copper-free water and dried over filter sheets in an airy room (temperature 30°C. to 35°C.) in such a way that no contamination might take place with copper from any outside sources. The nuts, cereals, and similar other products were directly weighed. Milk was collected in a glass vessel previously washed and dried carefully.

EXPERIMENTAL.

The metal has been determined by the colorimetric method of Biazzo (1926) as modified by Elvehjem and Lindow (1929). The samples were, however, ashed in silica basins and the nitric acid solution added during the process of analysis was finally neutralized by ammonium hydroxide prepared by passing ammonia gas into distilled water obtained in the laboratory by distilling in a set with all-glass joints. For the formation of the coloured complex the glacial acetic acid and potassium thiocyanate used were of reagent quality, and the pyridine (extra pure) was freshly distilled from a standard glass-joint set before use. After the extraction of the green copper-thiocyanate pyridine compound, the container was left aside in the dark for a few minutes for complete separation of the chloroform layer. It was subsequently compared with a standard in a Klett colorimeter. The result was taken from an average of two analyses in the case of vegetables and fishes, and of three in the case of cereals and pulses. Milk from different sources was again analysed several times.

The Biazzo method was followed mainly for the availability of the reagents in question. As most of the materials excepting certain leafy vegetables contain copper in amounts about 1 part per million and as the experiments were carried out carefully with proper control, there should be no inaccuracy from the practical point of view. In assaying of the metal from milk, the case may, however, be different and it would be better if this work be undertaken in any other laboratory more devoted to analytical work and where the 'dithiazone' extraction method of Fischer (1934), as modified by Sylvester and Lampitt (1935) and recommended by Coulson (1936), may be followed. In our hands cow's milk is found to contain 0.15 mg. to 0.25 mg. per litre. Steenbock *et al.* (1929) find cow's milk to contain 0.12 mg. to 0.2 mg. of copper per litre. The Table below gives the results of the assay:—

TABLE.

FOODSTUFF.			Percentage of copper in mg.
Bengali name.	English name.	Botanical name.	
Chaul (siddha)	Rice, parboiled, milled	<i>Oryza sativa</i>	0.20
" "	Rice, parboiled, home-pounded.	"	0.335
" (atap) ..	Rice, raw, milled	"	0.31

TABLE—contd.

FOODSTUFF.			Percentage of copper in mg.
Bengali name.	English name.	Botanical name.	
Chira	Rice, rolled	<i>Oryza sativa</i>	0·345
Moori	Rice, parched	"	0·297
Atta	Wheat, whole	<i>Triticum vulgare</i>	0·54
Flour	Wheat, milled	"	0·195
Suji	Semolina	"	0·170
Rooti	Bread, brown	"	0·25
Sagu	Sago	<i>Metroxylon sago</i>	0·263
Barley	Barley, milled	<i>Hordeum vulgare</i>	0·351
Jab, powder	Barley, home-pounded	"	0·628
Pala (sati)	<i>Curcuma zeodoria</i>	0·383
Mung dhal (kancha)	Black gram (unroasted)	<i>Phaseolus mungo</i>	0·706
Mung (roasted)	Black gram (roasted)	"	0·624
Mash kalai	Green gram	<i>Phaseolus radiatus</i>	0·437
Chhola	Bengal gram	<i>Cicer arietinum</i>	0·946
Moosur dhal	Lentil	<i>Lens esculenta</i>	0·70
Arhar dhal	Red gram	<i>Cajanus indicus</i>	0·73
.. ..	Soya bean (black)	<i>Glycine hispida</i>	0·827
.. ..	" (white, small)	"	0·928
Hingche	<i>Enhydra fluctuans</i>	0·231
Kalmi shak	<i>Ipomæa reptans</i>	0·191
Palong shak	Spinach	<i>Spinacia oleracea</i>	0·049
Kôpi (bandha)	Cabbage	<i>Brassica oleracea</i>	0·051
Puin shak	<i>Bassela cardifolia</i>	0·122
Betel leaves	<i>Piper betel</i>	0·263
Alu	Potato	<i>Solanum tuberosum</i>	0·24
Patôl	<i>Trichosanthes diœcia</i>	0·114
Dharôs	Lady's finger	<i>Hibiscus esculentus</i>	0·075

TABLE—concl'd.

FOODSTUFF.			Percentage of copper in mg.
Bengali name.	English name.	Botanical name.	
Begun	Brinjal	<i>Solanum melongena</i>	0.172
Kancha kala	Plantain, green	<i>Musa paradisiaca</i>	0.10
Kancha lanka	Peppers, green	<i>Piper nigrum</i>	0.13
Martaman kala	Banana, ripe	<i>Musa sapientum</i>	0.30
Champa kala	"	"	0.17
Paniphal	Water chestnut	<i>Trapa bispinosa</i>	0.28
Pesta	Pistachio nut	<i>Pistacia vera</i>	1.06
Badam	Almond	<i>Prunas amygdalis</i>	1.15
China badam	Ground nut	<i>Arachis hypogea</i>	0.90
Methi	Fenugreek seeds	<i>Trigonella fœnumgræcum</i>	0.79
Sarisa	Mustard seeds	<i>Brassica juncea</i>	0.65
Doodh (a)	Cow's milk	..	0.015
" (b)	"	..	0.0178
" (c)	"	..	0.0242
Egg (fowl)	0.18
Chingree	Prawn	..	0.77
Katla	Cutla fish	..	0.291
Ilish	Hilsa	<i>Clupea ilisa</i>	0.36

DISCUSSION.

These results are being found to agree on the whole very well with those of Lindow, Elvehjem and Peterson (1929). From the amount of copper present in different foodstuffs, it is evident that an average adult diet consisting of cereals, pulses, nuts, and fishes would afford about 3 mg. of copper per day, whereas according to Chou and Adolph (1935) the daily copper requirement for a man is 2 mg. Of course all the copper of our diet may not always be available (Sherman *et al.*, 1934). But there exists (Sachs *et al.*, 1935; Hahn and Fairman, 1936) an inverse relationship between iron and copper; that is when the iron content of the blood decreases, the copper content increases. Consequently a deficiency of copper in adults seems to be improbable even in cases of anæmias. Indeed Duckles, Willis and Elvehjem (1937) have recently shown that hypochromic anæmia of college women responds

as well to iron alone as to iron with copper. The daily copper requirement for normal children according to Daniels and Wright (1934) is 0.1 mg. per kg. of body-weight per day. But cow's milk is found to contain only 0.19 mg. per litre. From this it is evident that a diet according to Holt and McIntosh (1933) for an infant (who is being artificially fed), 3 months old weighing 12 lb., consisting of 21 oz. of milk a day, would not afford more than 0.15 mg. of copper; whereas the infant requires about 0.54 mg. of copper for its proper growth. The requirement is further considered to increase in cases of anæmic infants (Marriott, 1935; Hawksley, 1934; Chou and Adolph, *loc. cit.*). This, in other words, points to the necessity of treating the infants with copper. Actually the metal supplemented with iron has been found to cause a better response in the regeneration of blood hæmoglobin in anæmic infants (Parsons, 1931; Lewis, 1931; Elvehjem *et al.*, 1937; Hutchison, 1938).

CONCLUSION.

1. A number of cereals, pulses, and a few other Indian foodstuffs have been investigated with reference to the copper content. The nuts, pulses, cereals, and fishes are found to have the highest amounts, whereas cow's milk is low in copper.

2. From the amount of copper that is present in different foodstuffs it seems improbable that there is any serious copper deficiency in any adult mixed diet.

3. From the low percentage of copper in cow's milk, the question arises whether a trace of copper salt should be added in order to enrich the infant dietary.

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LEAD IN HUMAN TISSUES.

BY

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IN a previous communication (Bagchi and Ganguly, 1937a) the result of an investigation about the lead content of urine and faeces of Indians was described. It has proved useful as an aid to diagnosis of chronic lead poisoning of industrial or occupational origin and in detecting malingerers who are coached to describe the symptoms of lead poisoning with utmost precision and who manage to pass in their faeces as much as 239 mg. of lead per kilo (Bagchi, 1938). The lead content of urine and faeces also gives an idea about the probable amount of lead intake and its concentration in the different organs of an individual.

In fatal cases of acute lead poisoning, either accidental or suicidal, or for purposes of procuring criminal abortion, the victims usually survive for a few days and develop various complications which subsequently carry them off. During this interval the elimination of lead goes on steadily and nothing is left in the stomach. The examination of the stomach contents which are always sent along with the viscera does not in most cases show any indication of acute lead poisoning. The analysis of the viscera (stomach, kidney, and portions of liver and small intestines which are usually sent for examination) shows only small quantities of lead—too small in comparison with cases of poisoning by arsenic and other metals. The maximum amount of lead found in the liver in one of our cases of acute poisoning by red-lead was 17.3 mg. per kilo (*vide* Table II), while in cases of arsenic poisoning 300 mg. or more of arsenic (as As_2O_3) are usually found in the same organ and in one case as much as 2,900 mg. could be detected (Bagchi and Ganguly, 1937b). In such cases of lead poisoning considerable hesitation is felt in giving any opinion as to the cause of death,

especially in view of the fact that nothing is known about the lead content of a normal liver or stomach or any other organ. The belief (though lately shaken off) that lead is not a normal constituent of human tissues was a source of further complications in medico-legal analysis. Presence of small quantities of lead in viscera was, therefore, liable to be construed either as an accident or as an indication of lead poisoning.

It has now been established that lead is normally present in human tissues as 'an inevitable consequence of life on a lead-bearing planet' (Kehoe *et al.*, 1933*a*) and attempts are also being made to find out its physiological significance, if any. In the absence of definite information as to the actual lead content of normal tissues of Indians, any opinion based on the findings of European and American workers is likely to be fallacious and this investigation was, therefore, taken up from a purely medico-legal point of view. An attempt has also been made to explain certain pathological conditions in the light of our findings regarding the amount of lead in different organs.

The viscera of about 900 individuals are received annually in this laboratory from four different provinces, viz., Bengal, Bihar, Orissa, and Assam, for medico-legal analysis. The materials for this investigation were supplied by the Police Surgeon of Calcutta, the Professor of Medical Jurisprudence, Calcutta Medical College, and by the Medical Officers in charge of the district and subdivisional towns. Every possible care was taken to see that a fairly representative selection was made. All hospital cases or cases with a terminal illness causing partial starvation and thereby a disturbed metabolism and an interference with the normal intake of lead were carefully excluded. Only the cases of rapidly fatal street accidents, shooting, stabbing, suicidal hanging, etc., were selected for this investigation.

The dithizone method of Lynch, Slater and Osler (1934), slightly modified to prevent the formation of an objectionable yellow colour which interferes with colorimetric matching and to get rid of the traces of oxidants likely to be present in the solution, was employed. The modification consists in treating the oxidation products with ammonium oxalate at two different stages of the analysis as casually suggested by the authors. This method has been found to provide a quite satisfactory means for determination of lead in all biological materials, especially bones and teeth. Although the spectrographic method is considered as the most accurate for determination of lead in biological materials (Boyd and De, 1933; Cholak, 1935), the dithizone method, which requires no expensive apparatus, has been shown by Cholak *et al.* (1937) to be equally satisfactory and both the methods 'may be used with equal assurance as to the reliability of the results in the estimation of amounts of lead in excess of one gamma'. The close agreement between the results of duplicate analysis and the satisfactory recovery of added quantities of lead in our hands demonstrate the accuracy and usefulness of the dithizone method for purposes of this investigation.

About 25 grammes of soft tissues were taken for each analysis and most of the determinations were made in duplicate. Experiments giving too high or too low results were repeated to discard or confirm them. Hair, teeth, nails, and bones were taken in small quantities (2 g. to 5 g.). Merck's 'guaranteed reagents' and Pyrex glassware carefully treated with hot nitric acid were used throughout the analysis. Blanks were performed with every new bottle of reagents and new set of glassware

and the results of analysis were corrected accordingly. Steps were taken to remove all sorts of dirt and contaminations from materials such as hair, nails, tooth, and skin. The results have been shown in the following tables in the convenient form of milligrams of lead (as Pb) per kilo of fresh materials:—

TABLE I.

Lead content of normal tissues.

The figures indicate milligrams of lead per kilo of fresh tissues.

Serial number.	Tissues.	Number of specimens examined.	QUANTITY OF LEAD FOUND IN THE TISSUES.		Average.	REMARKS.
			Minimum.	Maximum.		
1	Liver ..	9	0.31	0.82	0.57	..
2	Kidney ..	8	0.37	0.71	0.50	..
3	Spleen ..	5	0.30	0.52	0.36	..
4	Stomach ..	8	0.20	0.60	0.41	..
5	Small intestine	6	0.20	0.60	0.38	..
6	Large intestine	5	0.30	0.68	0.55	..
7	Heart ..	5	0.45	0.75	0.56	..
8	Lungs ..	5	0.30	0.60	0.45	..
9	Blood ..	6	0.11	0.45	0.24	..
10	Thyroid ..	4	0.40	0.60	0.48	..
11	Ovary ..	6	<i>Nil</i>	<i>Nil</i>	<i>Nil</i>	..
12	Placenta ..	6	0.30	0.36	0.32	..
13	Uterus ..	5	0.05	0.47	0.28	..
14	Testes ..	4	0.30	0.40	0.34	..
15	Brain ..	5	<i>Nil</i>	0.10	0.073	<i>Nil</i> , 0.075 mg., 0.09 mg., 0.1 mg., and 0.1 mg. respectively.
16	Muscle ..	5	0.14	0.70	0.33	..
17	Skin ..	3	0.33	0.50	0.44	With its hair but without the sub-cutaneous fatty tissue.
18	Scalp ..	2	1.0	1.2	1.1	After complete depilation.
19	Fat ..	3	<i>Nil</i>	<i>Nil</i>	<i>Nil</i>	..
20	Cartilage ..	5	0.45	3.25	1.25	From ribs and sternum.
21	Bone ..	8	6.8	39.3	15.8	Rib 8.2 mg. to 8.5 mg. Tibia 6.8 mg. to 14.5 mg. Femur 12 mg. to 22.6 mg. Humerus 39.3 mg. Skull bone 14.8 mg.
22	Tooth ..	6	15.5	23.0	20.7	Maximum of incisors 19.0 mg. and of tricuspid 23.0 mg.
23	Hair ..	88	6	508.0	80.9	..
24	Nails ..	3	11.3	12.7	12.0	..

TABLE II.
Lead content of tissues of two cases of acute lead poisoning by red-lead.
Milligrams per kilo.

Number.	Age, sex, etc.	Liver.	Kidney.	Stomach.	Spleen.	Small intestine.	Urine.	REMARKS.
1	Hindu male aged 26	..	4.6	3.40	2.1	Admitted in hospital with jaundice and epigastric pain. Became unconscious and died after 34 hours.
2	Hindu male aged 23	..	2.8	1.46	..	1.33	1.65	Admitted in hospital in semi-conscious state and died in a few hours.

TABLE III.

Lead content of fetal tissues.

Milligrams per kilo.

Number.	Description of fetus.	TISSUES.												REMARKS.	
		Liver.	Kidney.	Lungs.	Heart.	Thymus.	Small intestine.	Large intestine.	Spleen.	Sternum.	Long bone.	Flat bone.	Bladder.		Brain.
1	Fœtus No. 1, five months.	0.28	0.06	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Obtained from the uterus of a woman who committed suicide.
2	Fœtus No. 2, seven months.	0.16	0.25*	Nil	Nil	Nil	0.07	0.1	0.05
3	Fœtus No. 3, nine months.	1.8	0.33	0.2	0.2	6.0	0.16	..
4	One month child	0.4	0.1	Nil	Nil	0.05	0.4	4.0	3.25	..	Nil	..
5	Fœtus No. 4, four months.														..

This fœtus (13 cm. in length), preserved in spirit for about a year, was divided into three portions, viz., (1) head and neck, (2) thorax and abdomen, and (3) upper and lower extremities and each portion was analysed separately. Total weight of the fœtus (spirit specimen) was 82 grammes and the total quantity of lead found in the entire fœtus was 0.021 mg., as shown below :—

(1) Head and neck (34 g.)—0.002 mg. of lead found = 0.059 mg. per kilo.
 (2) Thorax and abdomen (38.5 g.)—0.003 mg. of lead found = 0.077 mg. per kilo.
 (3) Extremities (9.5 g.)—0.016 mg. of lead found = 1.684 mg. per kilo.

* With adrenal glands.

TABLE IV.

Lead content of tissues of persons with history of abnormal lead exposure.
 Milligrams per kilo.

Number.	Tissues.	I. Hindu female aged 22, death due to extensive burns.	II. Hindu male aged 40, death due to fracture of skull.	III. Hindu male aged 26, death due to shock from injury.
1	Liver ..	0.98	3.60	1.5
2	Kidney ..	4.0	3.90	..
3	Heart ..	0.24	0.90	..
4	Lungs ..	0.60	1.09	3.6
5	Blood ..	0.38
6	Spleen ..	0.72	1.87	..
7	Stomach ..	2.20	1.10	..
8	Small intestine ..	1.50	0.90	1.2
9	Large intestine ..	1.87	1.2	..
10	Thyroid ..	0.46
11	Ovary ..	0.17
12	Uterus ..	0.60
13	Testis	1.20	..
14	Brain ..	0.90	0.75	..
15	Cartilage ..	1.70
16	Skin	0.75	..
17	Hair ..	30.6

TABLE V.

A comparative statement showing the maximum amount of lead found in normal tissues in different nationalities.

Milligrams per kilo.

Number.	Tissues.	INDIAN. (Taken from Table I of this paper).	AMERICAN. (Recorded by Kehoe <i>et al.</i> , 1933 <i>b</i>).	BRITISH. (Recorded by Tompsett and Anderson, 1935).
1	Liver ..	0.82	0.80	4.63
2	Kidney ..	0.71	0.70	2.60
3	Heart ..	0.75	Trace	..
4	Lungs ..	0.60	0.30	0.88
5	Intestines ..	0.68	0.20	..
6	Spleen ..	0.52	Trace	5.9
7	Cartilage ..	3.25	2.60	..
8	Skin {	0.50 (skin)
		1.20 (scalp)	1.30	..
9	Brain ..	0.10	0.10	0.72
10	Bones {	8.5 (rib)	11.4 (long bone)	12.9 (rib).
		14.86 (flat)	11.1 (flat).	..

DISCUSSION.

It will be noticed in Table V that the lead content of normal tissues of Indians is practically of the same general magnitude as recorded by Kehoe *et al.* (1933*b*) in connection with two healthy cases, one of them being a negro. On the other hand our findings are much less than those recorded by Tompsett and Anderson (1935) who conducted the investigation on necropsy materials obtained not from healthy men but from patients who died as a result of varieties of terminal illness in the Glasgow Royal Infirmary. Such well-marked differences in the findings may be accounted for by the various aspects of normal lead absorption. The lead content of tap water of Glasgow is 0.03 mg. per litre (Tompsett, 1936*a*) against the maximum of 0.002 mg. found in the samples of water collected from different sources in Calcutta and its neighbourhood. The intake of lead in the food also differs in various respects. The nature and number of meals and the quality and quantity of food materials which are taken by the middle-class Indians, not to speak of the

THE ACTION OF LEUCOPOIETIC DRUGS*.

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EVER since it has been known that the neutrophil leucocytes are the fighting forces of the body, attempts have been made to find a drug which is capable of calling forth young cells from the marrow. Leucocytosis due to an increase of neutrophils has been observed with a number of drugs but rarely has any attempt been made to find out if the leucocytosis was due simply to mobilization of cells which normally stagnated in the viscera or to the actual proliferation of new cells in the bone-marrow.

I have, therefore, carried out experiments on rabbits with pharmacological agents reputed to be leucopoietic, to find out their mode of action and, if possible, to assess their value as leucopoietic agents. The drugs tested may be classified in the following groups:—

- (a) Substances generally reputed to be leucopoietic, e.g., pentnucleotide, sodium nucleinate, and liver extract.
- (b) Toxic substances, e.g., colchicine.
- (c) Sympathetic stimulants, e.g., adrenaline chloride.
- (d) Parasympathetic stimulants, e.g., acetyl-choline and carbaminoyl-choline ('Doryl', Merck).
- (e) Parasympathetic depressants, e.g., atropine.

I shall first briefly describe the methods and technique employed and then describe the action of each drug separately, and finally sum up the results obtained with the different drugs.

* This work was carried out by me during the year 1936 at the Medical Professorial Unit, St. Bartholomew's Hospital, London, while holding a Rockefeller Fellowship from the School of Tropical Medicine, Calcutta.

EXPERIMENTAL PROCEDURE.

A. Experimental animals.

Rabbits weighing about 2,000 g. to 3,000 g. and mostly over one year old (a few younger were between the ages of six months and one year) were used for the experiments. The blood was examined several times before any experiment was carried out and only animals with a steady total white cell count of not more than 10,000 to 12,000 per c.mm. were used. Sometimes the same animal was used for different experiments but the interval between two experiments was never less than two weeks and most often about four weeks.

B. Collection of blood.

The rabbits were placed in a wooden cage so constructed that the whole body remained inside the box and only the head peeped out, thus minimizing the chances of struggling during the collection of blood. All samples were collected at about 10 a.m. When repeated daily injections were given, blood was always first collected before the injection was given.

C. Examination of blood.

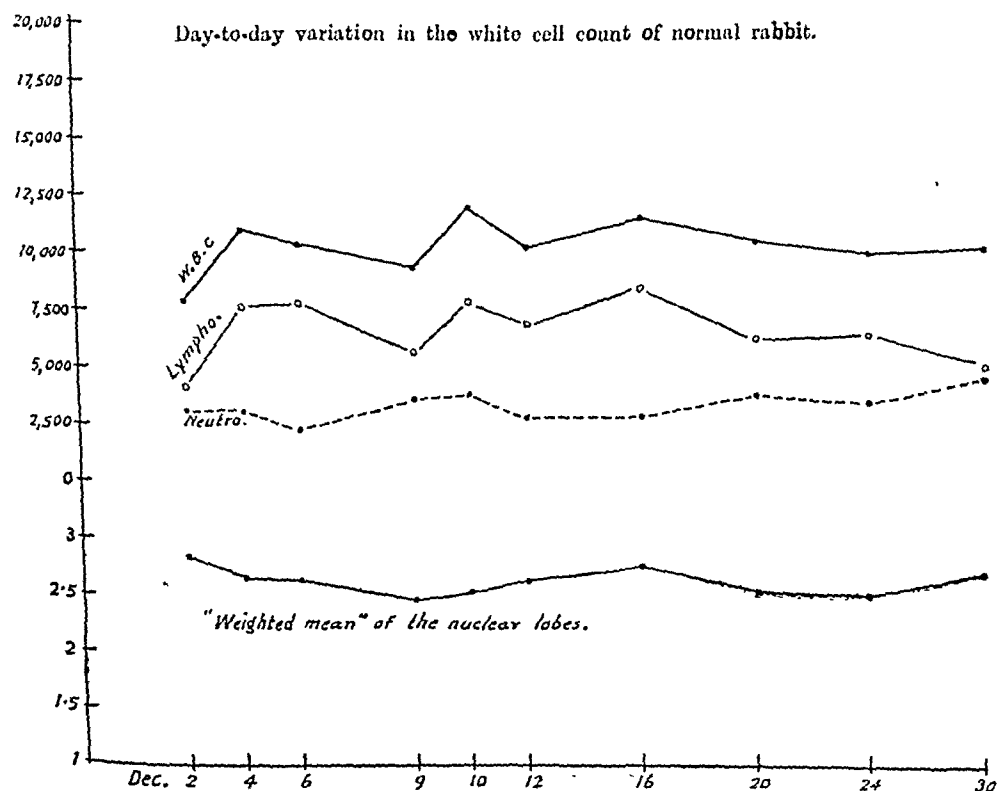
1. *Total white blood cell count.*—Blood was taken up to the 0.5 mark of the white pipette and diluted to 1 in 20. Using a 1/6 objective, the squares in the centre of the field of the counting chamber were focussed and the tube was drawn out until the field measured eight-twentieths of a millimetre, i.e., eight times the length of the side of a small square. The area of the field was now almost equal to the area of 50 small squares. The count was made by counting the number of cells in 40 different fields. The calculation was made in the usual way, remembering that each field contains 50 squares and that the volume of each square is 1/4,000 c.mm. Thus, if there are 200 cells in 40 fields and the dilution is 1 in 20 the number of cells per c.mm. is $\frac{4,000 \times 20}{50 \times 40} \times 200$, i.e., 8,000 and the same result is obtained by multiplying the number of cells by 40 if the dilution is 1 in 20 or by 20 if the dilution is only 1 in 10. In all cases the cells in both sides of a double counting chamber were counted and the average of the two counts taken. This method, though more laborious than the usual method of counting, seems to be more accurate, as the cells of a very large area are counted and the multiplying factor is only 40 or 20.

2. *Differential white blood cell count.*—A small drop of blood from the same drop from which the pipette for the total white count was filled was taken with a small platinum loop on a clean slide by a second person and the whole drop was used to make a thin smear occupying the middle of the slide. The smear was stained by a modification of the May-Grunwald and Giemsa method, with which the different types of granulations and the lobulation of the polymorphonuclear neutrophils were very distinct and the Arneth count was carried out with great ease.

In order to replace the usual expression for distribution of the cells in the Arneth count by a single figure, Ponder and Flint (1926) advocated taking the weighted mean of the observations by multiplying the cells of class I by 1, those of class II by 2, of class III by 3, and so on—the results being finally summed up and divided by the total number of cells counted. This method was used as it has the advantage that it can be graphically recorded and fluctuations however small can be noticed. A low value indicates the presence of larger numbers of young cells of classes I and II in the peripheral circulation and as such is of great value in distinguishing whether the leucocytosis is merely due to re-distribution of cells from stagnated viscera or is due to young cells from the bone-marrow.

3. *Variation in the white cell count in normal rabbits.*—A day-to-day count on a number of normal rabbits was done for over a month and a sample for a particular animal is shown in Chart 1, Table I. There are only slight variations in the

CHART 1.



total and differential count as well as in the nuclear lobe count as shown in the weighted mean. The diurnal variation of the count was also noted in a few cases

TABLE I.

Day-to-day variation in the white cell count of a normal rabbit.

Date.	W. B. C. total.	NEUTROPHIL.		'Weighted mean.'	LYMPHOCYTE.	
		Per cent.	Total.		Per cent.	Total.
2-12-35 ..	7,880	39.0	3,073	2.83	51.5	4,058
4-12-35 ..	11,000	28.0	3,080	2.66	69.0	7,590
6-12-35 ..	10,300	22.6	2,327	2.62	74.0	7,622
9-12-35 ..	9,240	38.0	3,511	2.47	60.0	5,544
10-12-35 ..	11,960	31.0	3,708	2.51	64.0	7,654
12-12-35 ..	10,080	28.5	2,873	2.61	68.0	6,354
16-12-35 ..	11,400	25.0	2,850	2.73	73.0	8,322
20-12-35 ..	10,360	35.0	3,626	2.51	58.0	6,000
24-12-35 ..	9,840	35.0	3,444	2.47	63.5	6,248
30-12-35 ..	9,920	45.0	4,464	2.65	50.0	4,960

under normal conditions and also after the subcutaneous and intravenous injections of fresh normal salt solution. In all cases (Chart 1-A, Table I-a) a slight afternoon rise in the total white cell count was noted but there was no appreciable change in the polymorph-lymphocyte proportion in the differential count nor was there any change in the weighted mean.

CHART I-A.

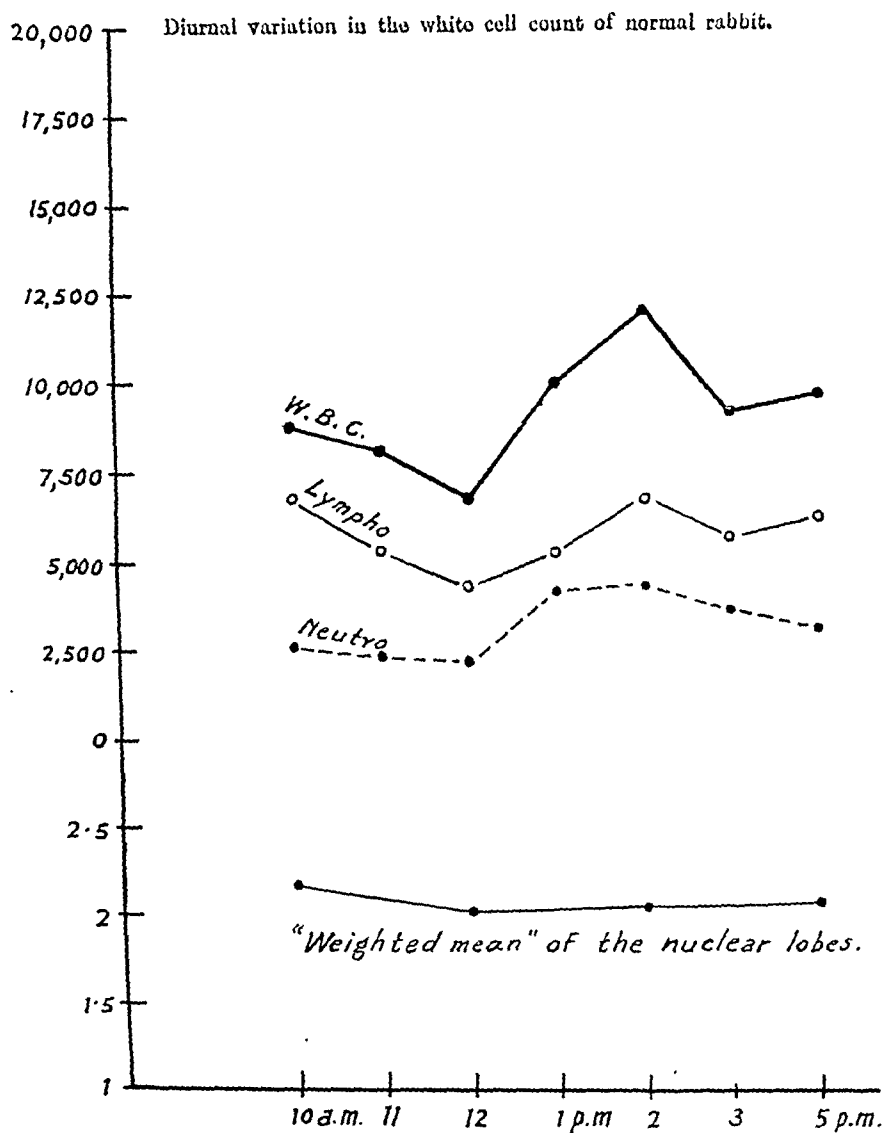


CHART 1-B.

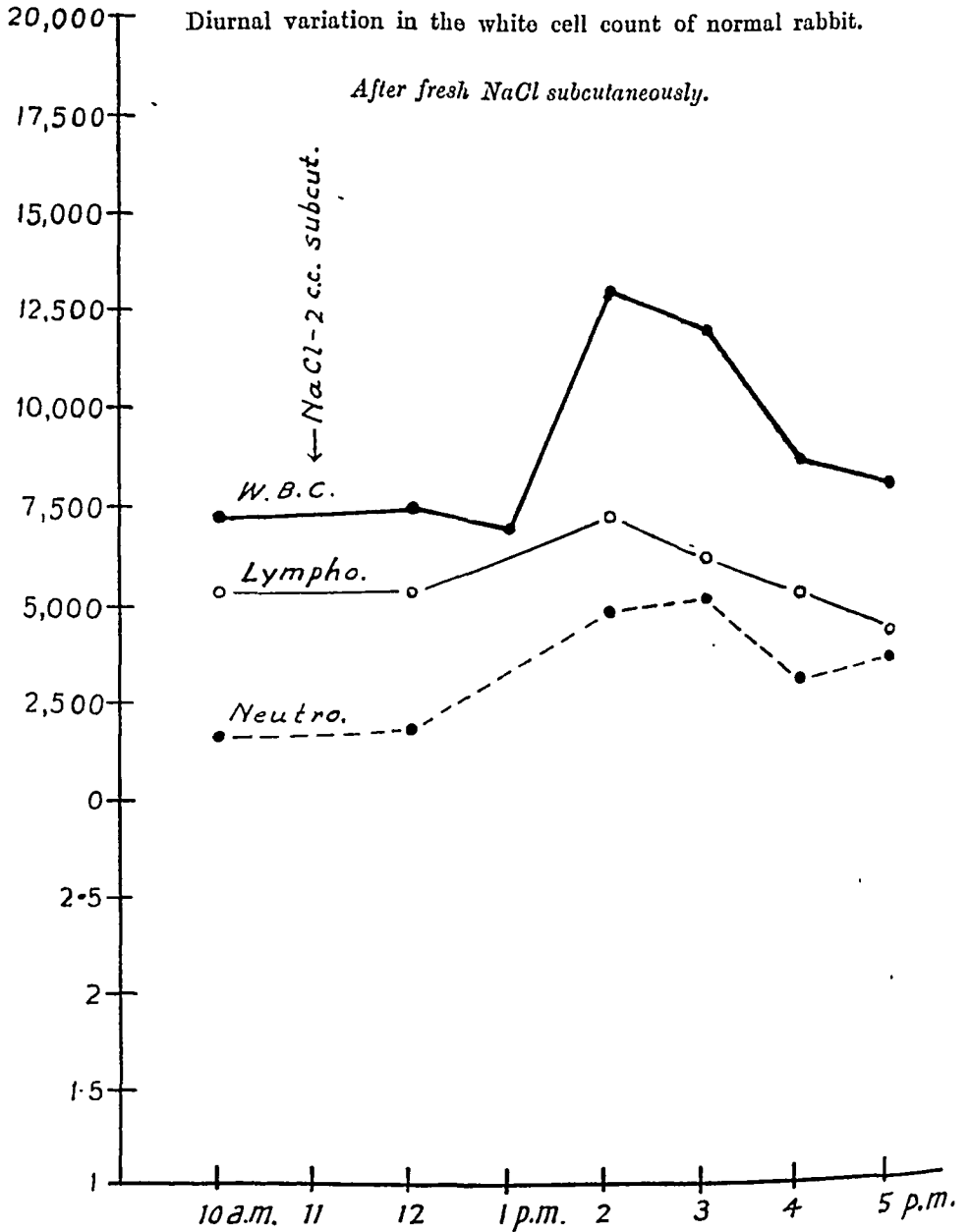


CHART 1-C.

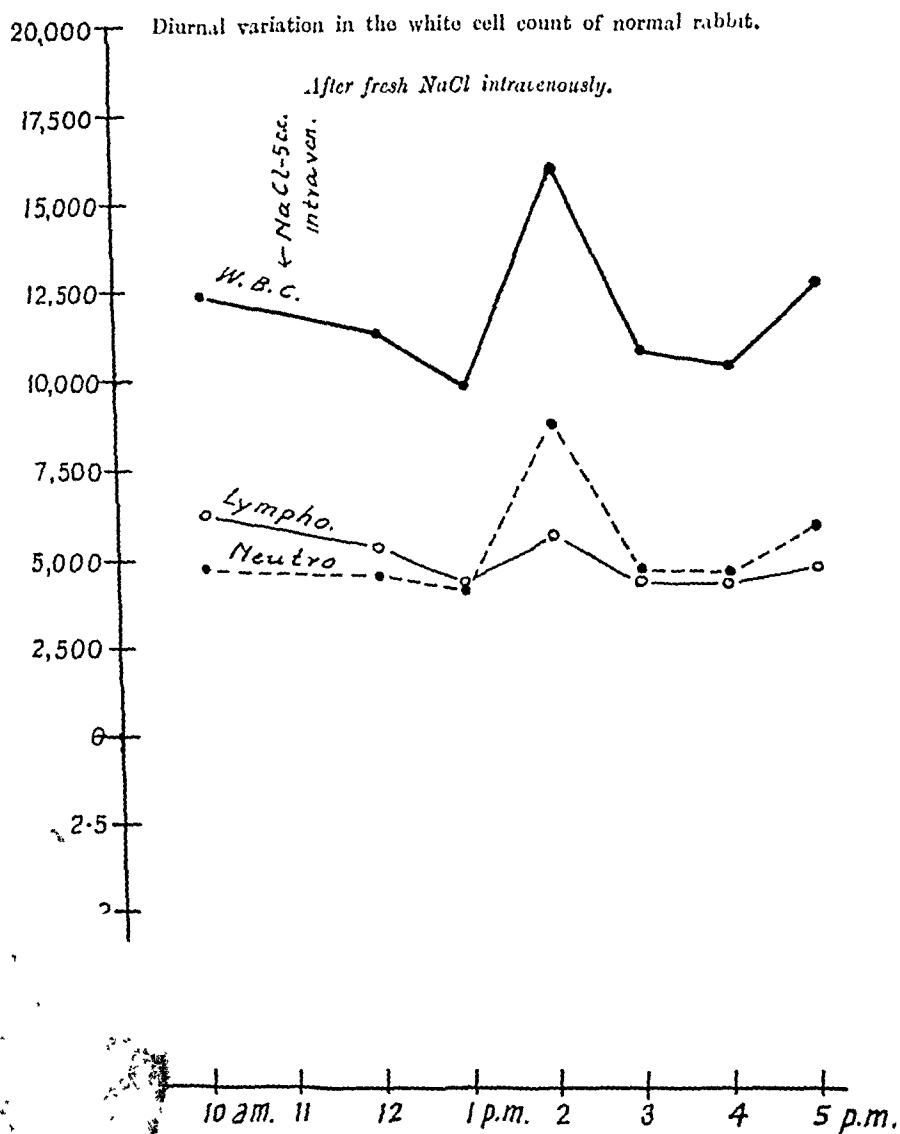


TABLE I-a.

Diurnal variation in the white cell count of a normal rabbit.

Time.	W. B. C. total.	NEUTROPHIL.		'Weighted mean.'	LYMPHOCYTE.	
		Per cent.	Total.		Per cent.	Total.
10 a.m. ..	8,800	30.0	2,640	2.19	67.0	6,896
11 „ ..	8,080	30.0	2,424	..	67.0	5,414
12 „ ..	6,800	33.0	2,244	2.01	65.0	4,420
1 p.m. ..	10,000	41.0	4,100	..	53.0	5,300
2 „ ..	12,000	37.0	4,440	2.04	67.0	6,840
3 „ ..	9,190	35.5	3,662	..	62.0	5,698
5 „ ..	9,680	32.0	3,088	2.08	64.0	6,195

TABLE I-b.

After injection of 2 c.c. of fresh normal saline subcut

Time.	W. B. C. total.	NEUTROPHIL.		'Weighted mean'
		Per cent.	Total.	
Before injection .. 10 a		22.0	1,610	
11		2 c.c. NaCl injected sub		
1 hour after injection 12		24.0	1,800	
2 hours „ „ 1 p.m		
3 „ „ „ 2 „		0	4,940	
4 „ „ „ 3 „			5,160	
5 „ „ „ 4 „			3,010	
6 „ „ „ 5 „			3,520	

OCYTE.

To

5

TABLE I-c.

After injection of 5 c.c. of normal saline intravenously.

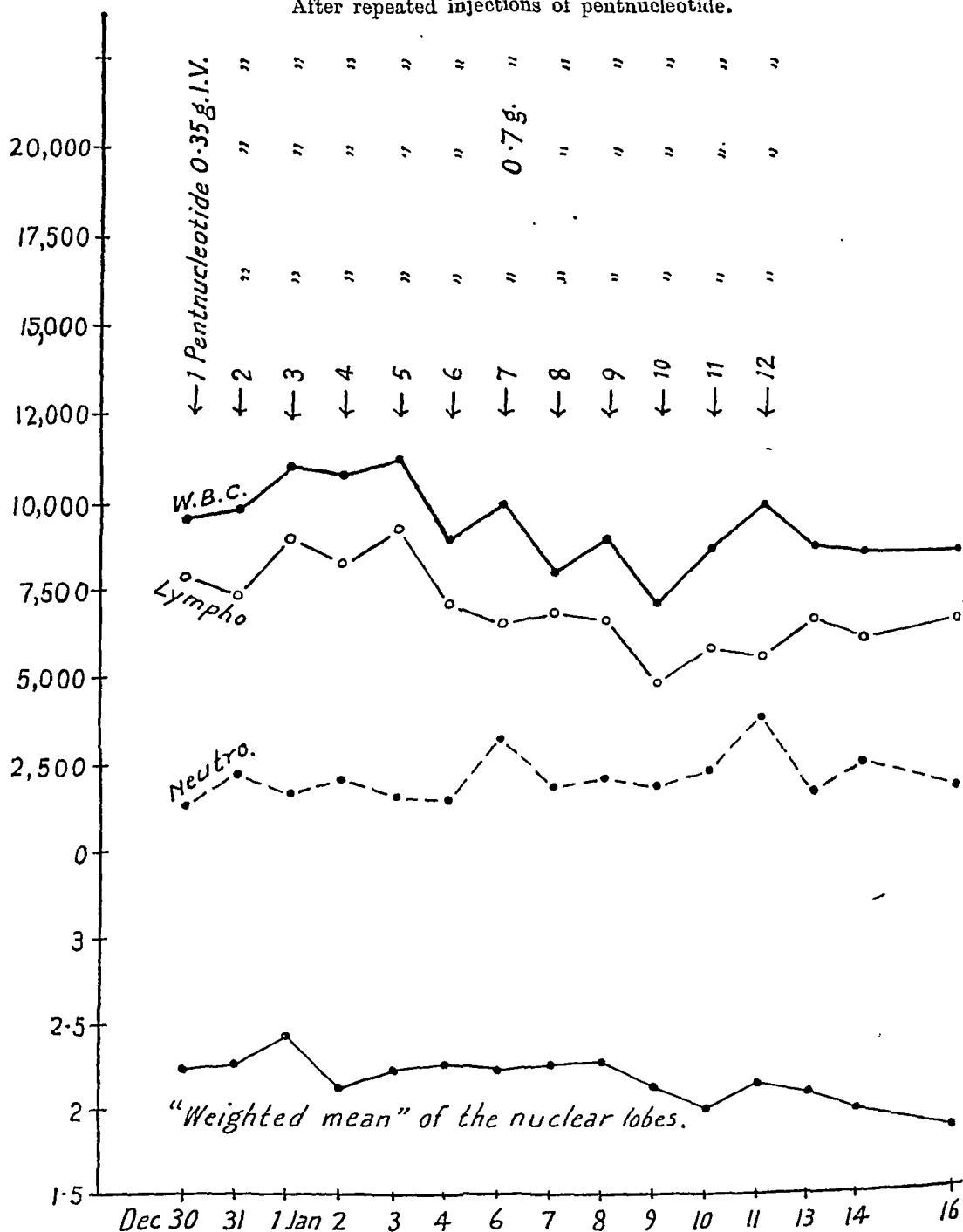
Time.	W. B. C. total.	NEUTROPHIL.		'Weighted mean.'	LYMPHOCYTE.	
		Per cent.	Total.		Per cent.	Total.
Before injection .. 10 a.m.	12,440	39.0	4,852	..	59.0	6,354
11 .. 5 c.c. NaCl injected intravenously.						
1 hour after injection 12 ..	11,400	40.0	4,560	..	48.0	5,472
2 hours 1 p.m.	9,400	46.0	4,326	..	47.0	4,418
3 2 ..	16,000	55.0	8,800	..	35.0	5,600
4 3 ..	10,800	44.0	4,752	..	41.0	4,428
5 4 ..	10,400	45.0	4,680	..	42.0	4,368
6 5 ..	12,600	47.5	5,985	..	38.0	4,788

1. PENTNUCLEOTIDE AND SODIUM NUCLEINATE.

Nuclein therapy was suggested purely on empiric grounds as early as 1893 to increase the germicidal power of the blood in bacterial diseases (Vaughan, Nory, and McClintock, 1893). Subsequent workers (Fox and Lynch, 1917; Neymann, 1917) found that the hypodermic injection of nucleoproteins, nucleins, and nucleic acid produces leucocytosis of short duration, mainly due to increase in the polymorphonuclear leucocytes. Fox and Lynch (*loc. cit.*) noted that the leucocytosis was preceded by an initial leucopenia. Sabin (1923) in the course of examination of normal living blood cells found 'non-motile' polymorphonuclear neutrophils which occurred in 'showers' in the peripheral circulation. Doan and Zerfas (1927) correlated these showers of non-motile leucocytes in human pathological conditions with subsequent increases of young motile neutrophils. Both these observations suggest that the 'zonal range' of the leucocytes is maintained by the disintegrating products of cells of the same type, and nuclear derivatives are an important component of these. The suggestion was strengthened by the subsequent finding of pentose-nucleotide in normal blood by Jackson (1924).

CHART 2-A.

After repeated injections of pentnucleotide.



at a low level. The neutrophils continued at a high level and the lymphocytes at a low level up to the sixth hour after the injection after which both returned to the pre-injection level at the 24th hour. An occasional myelocyte was found at the second, third, and fourth hour after injection, i.e., at the height of neutrophil

TABLE II.

After single injection of 0.7 g. of pentnucleotide intravenously.

Time.	W. B. C. total.	NEUTROPHILS.		'Weighted mean.'	LYMPHOCYTE.		Immature cell, per cent.
		Per cent.	Total.		Per cent.	Total.	
Before injection ..	11,600	40.0	4,640	2.49	55.0	6,380	Nil.
$\frac{1}{2}$ hour after injection ..	10,000	46.0	4,600	2.62	49.0	4,900	Nil.
1 " " " ..	8,000	62.0	4,960	2.49	32.0	2,560	Nil.
1 $\frac{1}{2}$ hours " " ..	15,400	79.0	12,166	2.43	18.0	2,670	Nil.
2 " " " ..	15,120	76.0	11,365	2.34	22.0	3,326	0.3
3 " " " ..	14,400	84.0	12,096	2.58	14.0	2,016	Nil.
4 " " " ..	12,050	75.0	9,000	2.63	19.0	2,280	Nil.
5 " " " ..	12,680	81.0	10,270	2.58	14.0	1,775	Nil.
6 " " " ..	11,720	74.0	8,576	2.51	22.0	2,578	Nil.
24 " " " ..	10,700	43.0	4,600	2.70	53.0	5,670	Nil.

TABLE II-a.

After repeated injections of pentnucleotide intravenously.

Date.	Amount, g.	W. B. C. total.	NEUTROPHILS.		'Weighted mean.'	LYMPHOCYTE.		Immature cell, per cent.
			Per cent.	Total.		Per cent.	Total.	
30-12-35 ..	0.35	9,600	15.0	1,450	2.23	83.0	7,960	<i>Nil.</i>
31-12-35 ..	0.35	9,920	23.0	2,282	2.29	75.0	7,440	<i>Nil.</i>
1-1-36 ..	0.35	11,120	26.0	1,779	2.16	82.0	9,118	Normo l.
2-1-36 ..	0.35	10,840	20.0	2,168	2.12	76.5	8,400	<i>Nil.</i>
3-1-36 ..	0.35	11,320	15.0	1,698	2.23	83.5	9,440	Normo l.
4-1-36 ..	0.35	9,000	17.0	1,530	2.28	80.0	7,200	<i>Nil.</i>
6-1-36 ..	0.7	10,000	33.0	3,300	2.23	65.0	6,500	<i>Nil.</i>
7-1-36 ..	0.7	8,000	24.0	1,920	2.27	74.0	6,920	<i>Nil.</i>
8-1-36 ..	0.7	9,080	24.0	2,179	2.29	73.0	6,620	Normo l.
9-1-36 ..	0.7	7,120	28.0	1,994	2.13	69.5	4,947	<i>Nil.</i>
10-1-36 ..	0.7	8,760	28.0	2,453	2.01	67.0	5,869	<i>Nil.</i>
11-1-36 ..	0.7	10,000	39.0	3,900	2.16	56.0	5,600	<i>Nil.</i>
13-1-36	8,840	20.0	1,780	2.10	77.0	6,776	<i>Nil.</i>
14-1-36	8,680	31.0	2,690	2.00	72.0	6,250	<i>Nil.</i>
16-1-36	8,640	21.0	1,814	1.87	77.0	6,653	<i>Nil.</i>

leucocytosis, but there was no increase in the staff or juvenile forms and the weighted mean remained unchanged throughout. After an intramuscular injection of 0.1

gramme of sodium nucleinate (Chart 3, Table III) there was slight leucopenia at the sixth hour and this was followed immediately by slight leucocytosis at the seventh hour which persisted up to the 24th hour, and returned to the level before injection at the 48th hour. The lymphocytes, however, began to fall from the first hour after injection attaining the lowest level at the sixth hour and returning to the level before injection after the 24th hour, while the neutrophils began to rise after the first hour, attaining the maximum height at the seventh hour and not coming back to the level before injection till the 36th hour. Even during the period of slight leucopenia at the sixth hour, the neutrophils were at a much higher point than at the pre-injection level. An occasional myelocyte was found during the height of leucocytosis but there was no increase in staff or juvenile forms and no change was found in the weighted mean.

CHART 3.

After injections of sodium nucleinate.

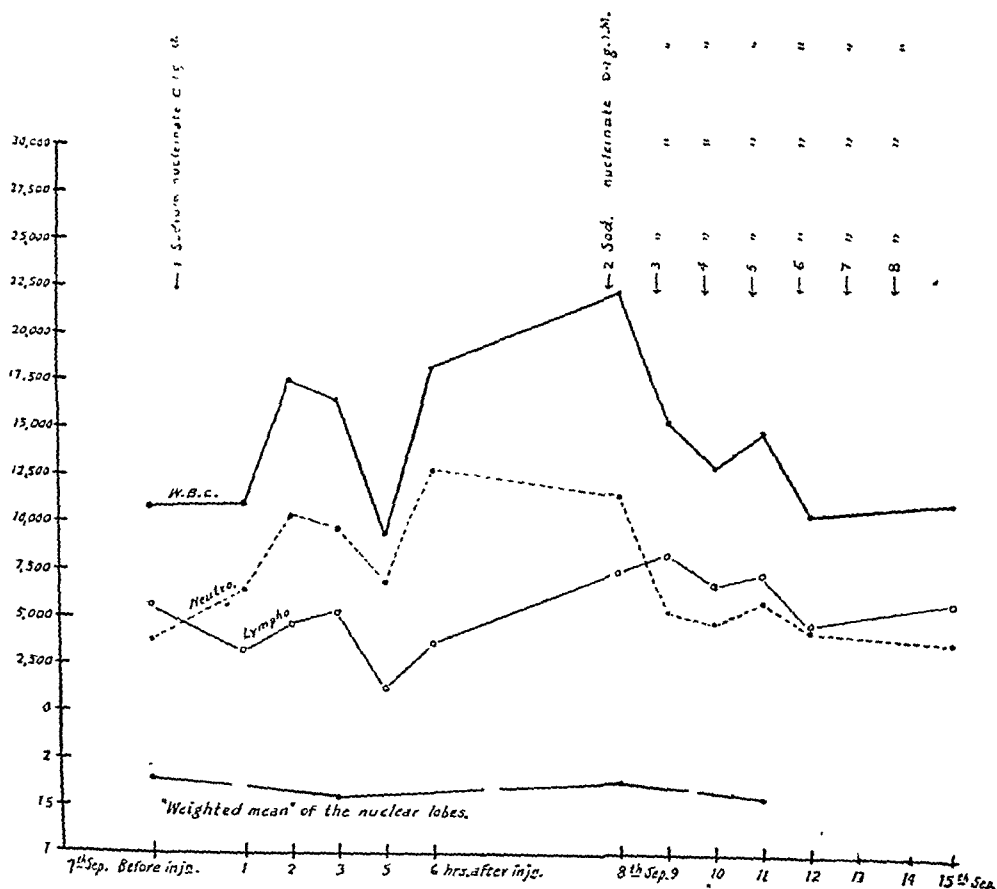


TABLE III.

After injections of sodium nucleinate.

Date.	Time.	Number and amount, g.	W. B. C. total.	NEUTROPHILS.		'Weighted mean.'	LYMPHOCYTE.		Immature cell, per cent.
				Per cent.	Total.		Per cent.	Total.	
7-9-36	10 a.m.	(1) 0.1	10,800	35.0	3,780	1.80	51.0	5,508	Nil.
	11 "	
	12 "		10,800	60.0	6,482	..	20.0	3,024	ct 1
	1 p.m.		17,400	59.0	10,266	..	27.0	4,658	" 0.3
	2 "		16,420	57.0	9,530	1.60	32.0	5,352	" 0.3
	3 "		9,240	75.0	6,930	..	15.0	1,385	Nil.
	4 "		18,000	70.0	12,600	..	20.0	3,600	Nil.
8-9-36	10 a.m.	(2) 0.1	22,000	52.0	11,440	1.76	34.0	7,480	ct 0.3 Normo 0.3
9-9-36	10 "	(3) 0.1	15,200	35.0	5,326	..	54.0	8,208	Nil.
10-9-36	10 "	(4) 0.1	12,800	37.0	4,736	1.60	53.0	6,784	Nil.
11-9-36	10 "	(5) 0.1	14,600	40.0	5,840	..	50.0	7,300	Nil.
12-9-36	10 "	(6) 0.1	10,400	42.0	4,388	..	44.0	4,576	Nil.
13-9-36	10 "	(7) 0.1
14-9-36	10 "	(8) 0.1
15-9-36	10 "	..	10,800	34.5	3,728	1.94	48.0	5,832	Nil.

Daily repeated injections.—Three rabbits weighing about 3 kilos each were injected intravenously with 0.35 gramme of pentnucleotide daily for six days (Chart 2-A, Table II-*a*) and then with 0.7 gramme daily for another six days. Altogether each animal had 12 consecutive injections of pentnucleotide or a total dose of 6.3 grammes of pentnucleotide. The animals tolerated the injections well without showing any reaction, kept quite fit and did not lose weight. During the whole period of the experiment there was no great variation either in the total or differential white cell count and the weighted mean remained almost constant, there being no increase in the staff or juvenile neutrophils. No immature white cell was found at any time during the experiment. The experiment was repeated in two rabbits with sodium nucleinate (Chart 3, Table III) which was given in a dose of 0.1 gramme intramuscularly daily for eight days. With this drug too no increase in the total white cells or in the neutrophils was observed, the weighted mean remained constant, there was no increase in staff or juvenile neutrophils, and no immature white cell was found at any time during the experiment.

Comment.—Leucocytosis, which was preceded by leucopenia and which was due to an increase in neutrophils, was seen after both pentnucleotide and sodium nucleinate. The leucocytosis observed after pentnucleotide, which was given by the intravenous route, appeared one hour after the injection and persisted only for three hours, whereas the leucocytosis observed after sodium nucleinate, which was given by the intramuscular route, did not manifest itself before the sixth hour but persisted for a longer period. Occasionally a myelocyte was found at the height of the leucocytosis after single injection of the drugs. Daily repeated injections of either drug, however, failed to maintain a sustained leucocytosis and no immature cell was found at any time during the period of observation. With both single and repeated injections the weighted mean of the nuclear lobes remained almost constant and there was no increase in staff neutrophils. These rabbits received 6.3 grammes of pentnucleotide, almost double the dose which was given by Doan to his rabbits, but still no striking granulocytosis was observed in the peripheral circulation nor was there any shift to the left of the Arneth count as has been claimed by Doan (1932*a*, *b*). It is seen from Doan's chart that a biopsy of bone-marrow was done on 24th January. Pentnucleotide injection was started on 26th January, only two days after the biopsy, and was given at irregular intervals, and the whole experiment was completed within one week from the date of the biopsy. It is also seen from the charts that the total leucocytes varied from 10,000 to 15,000 per c.mm. and the total neutrophils from 3,500 to 7,200 and not from 2,700 to 8,200 per c.mm. as is claimed by Doan, if, as is only fair, the initial count be taken before the biopsy of the bone-marrow and not after it. It is further to be noted that the maximum leucocytosis in most cases came shortly after the injections were given.

From my own experiments and from the analysis of Doan's chart, there is strong reason to suppose that the leucocytosis observed by Doan was well within the normal range of variation. It was due mostly to re-distribution of cells but partly to the inflammatory process following biopsy, and the latter was enough to account for the shift to the left in the Arneth count. This view is further supported by the absence of any immature white cells in the peripheral circulation. The leucocytosis observed after single doses of pentnucleotide and sodium nucleinate may be due

to a certain extent to a chemotactic effect of the drug on normal myeloid foci, as is evident from the findings of an occasional myelocyte at the height of leucocytosis, but in the main it is due to mobilization of cells stagnating in the viscera. This is almost conclusively proved by the constancy in the weighted mean and the absence of any increase in staff forms. The absence of any sustained leucocytosis after repeated injections of either drug confirms the view that they are ineffective in inducing proliferation of the leucopoietic tissues.

2. LIVER EXTRACT.

Most of the experiments reported on the action of preparations of liver on the leucopoietic system were carried out on human beings—healthy volunteers and patients in hospital—and though human beings under basal condition are less likely than animals to show variations in total and differential white cell count, still reports on the effect of the drug are rather conflicting. Thus, Cornell (1928) who gave 240 grammes of liver for four weeks to four normal persons, and Watkins *et al.* (1928) who gave 300 grammes of liver for 10 days, did not find any change either in the total or differential white cell count. Powers and Murphy (1933) found that the injection of concentrated liver extract in normal individuals was followed by neutrophil leucocytosis which was maximal in six to seven hours. Meyer, Middleton and Thewlis (1934) found that parenteral administration of concentrated liver extract was ordinarily attended by an early fall in the white cell count and subsequently by constant leucocytosis due to increase in the neutrophils in four normal and three abnormal cases. The maximal count was reached three hours after intravenous injection and five hours or later after intramuscular injection. Powers (1935) repeated his experiments not only to confirm his previous results but also to determine whether the attendant leucocytosis was due to the liberation of mature neutrophils, juvenile forms, or both. By injecting 3 c.c. of liver extract intramuscularly in seven healthy persons, he again found a neutrophilic leucocytosis about six hours after injection, which was due mainly to increase in the juvenile forms, and he concluded that the leucocytosis induced by the intramuscular injection of liver extract is due either to direct or indirect stimulation of the marrow.

In the series of experiments that were performed by me with liver extract, two rabbits were injected intravenously with 4 c.c. and 8 c.c. respectively of Neo-Hepatex (Evans) and the blood examined at short intervals up to six hours after the injection (Chart 4, Table IV). In both the animals a definite leucocytosis, due entirely to an increase in the neutrophils, was observed—the maximal increase was more pronounced and was noticed after three and a half hours after 8 c.c. injection, while maximal increase was less pronounced and manifested itself after four and a half hours after 4 c.c. injection of the drug. A slight fall in the weighted mean of the Arneth count due to increase in the staff forms was observed in both cases but no immature white cells were found at any time in either animal. A slight leucopenia due to decrease of both neutrophils and lymphocytes was noted 15 minutes after 8 c.c. injection and this leucopenia continued up to one hour. Thus, the results with single injection of liver extract corroborate in the main the findings of Powers and Murphy (*loc. cit.*), Meyer, Middleton and Thewlis (*loc. cit.*), and Powers (*loc. cit.*).

CHART 4.

After single injection of 8 c.c. of liver extract (Neo-Hepatex).

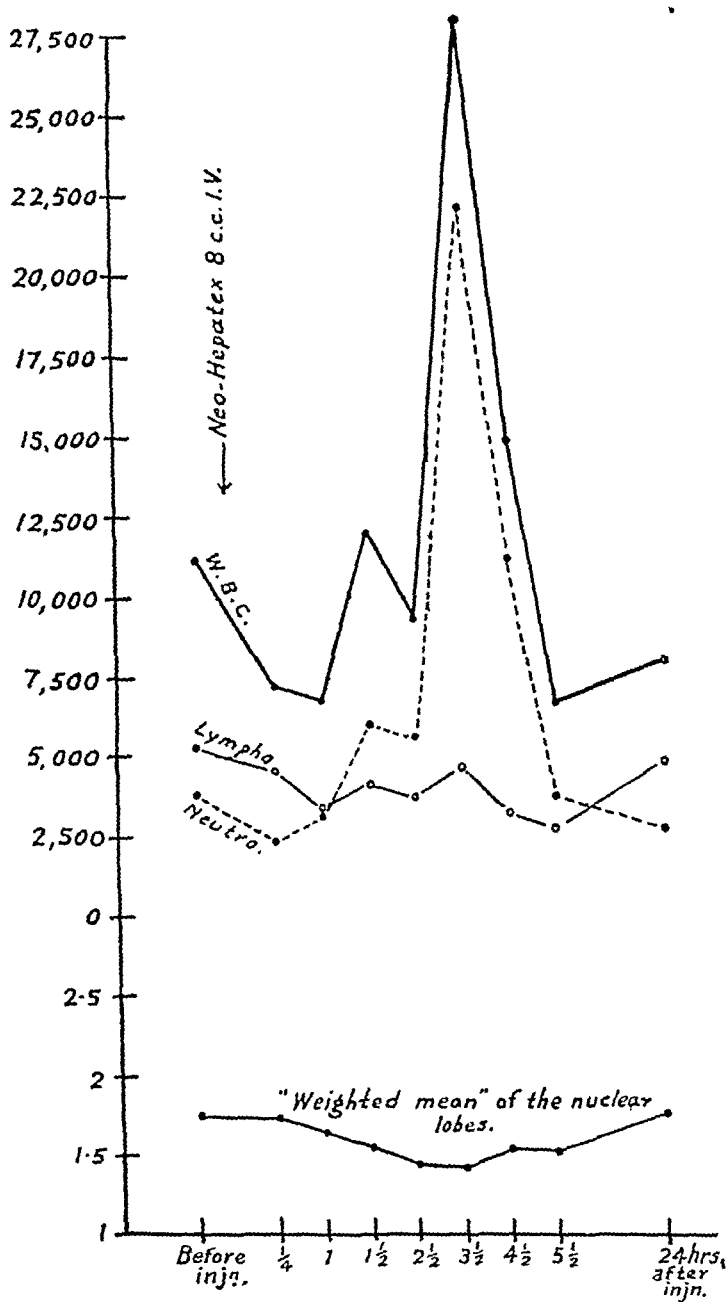


TABLE IV.

After single injection of 8 c.c. of Neo-Hepatex intravenously.

Time.	W. B. C. total.	NEUTROPHILS.		'Weighted mean.'	LYMPHOCTE.		Immature cell, per cent.
		Per cent.	Total.		Per cent.	Total.	
Before injection ..	11,150	35.0	3,902	1.76	48.0	5,352	Nil.
$\frac{1}{4}$ hour after injection ..	7,280	33.5	2,439	1.74	62.0	4,513	Nil.
1 " " " ..	6,880	46.0	3,165	1.65	50.5	3,474	Nil.
1 $\frac{1}{2}$ hours " " ..	12,000	56.0	6,000	1.56	34.0	4,080	Nil.
2 $\frac{1}{2}$ " " " ..	9,400	59.5	5,593	1.45	39.5	3,713	Nil.
3 $\frac{1}{2}$ " " " ..	28,000	79.0	22,120	1.43	16.5	4,620	Nil.
4 $\frac{1}{2}$ " " " ..	14,880	75.0	11,160	1.54	22.0	3,273	Nil.
5 $\frac{1}{2}$ " " " ..	6,700	56.5	3,785	1.52	41.0	2,747	Nil.
24 " " " ..	8,000	34.0	2,720	1.78	60.0	4,800	Nil.

Repeated injections.—It was now decided to observe the effects of the repeated injections of liver extract in normal rabbits to find out if it was capable of producing and maintaining a sustained leucocytosis. With this object in view two rabbits were subjected to repeated daily injections of liver extract—in one case by the intravenous route in doses of 2 c.c. daily for 14 days (Chart 4-A, Table IV-a), in the other by the intramuscular route in doses of 4 c.c. daily for 17 days. A definite prolonged leucocytosis due to increase of neutrophils was never observed in either case, rather a slight lymphocytosis for a short period was observed for the first few days when the injection was given intramuscularly. The 'weighted mean' in both instances was almost constant and no immature white cell was observed at any time during the period of the experiment. The red cells and hæmoglobin remained almost constant throughout the experiment. A rise in the reticulocyte count was, however, noticed in both animals from the seventh injection onwards. Both the animals were in good health and did not lose any weight during the whole period of the experiment. Both the animals were killed after the experiment and the examination of the bone-marrow revealed a very cellular condition of the marrow with leucoblastic areas slightly in excess of erythroblastic areas. It is probable that the reticulocytosis and the hyperplasia of the bone-marrow were merely the result of repeated examinations of the blood, as the amount lost in this way may be significant in a small animal like the rabbit.

CHART 4-A.

After repeated injections of liver extract (Neo-Hepatex).

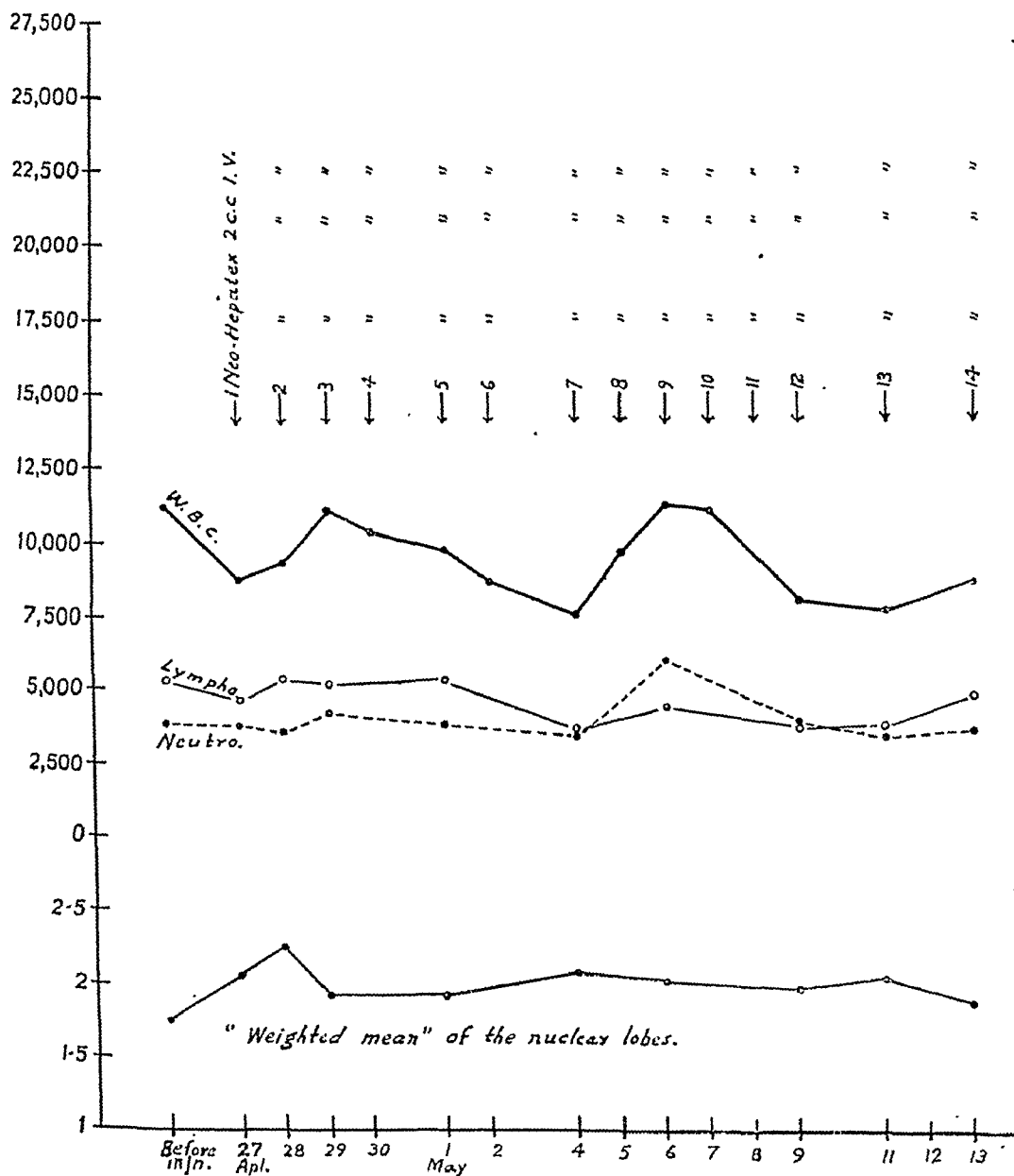


TABLE IV-a.

After repeated injections of Neo-Hepatex intravenously.

Date.	Number and amount, c.c.	W. B. C. total.	NEUTROPHILS.		'Weighted mean.'	LYMPHOCYTE.		Immature cell, per cent.
			Per cent.	Total.		Per cent.	Total.	
27-4-36 ..	(1) 2	8,800	44·0	3,872	2·06	53·0	4,664	<i>Nil.</i>
28-4-36 ..	(2) 2	9,300	39·0	3,534	2·25	57·0	5,301	<i>Nil.</i>
29-4-36 ..	(3) 2	11,000	40·0	4,840	1·92	49·0	5,390	<i>Nil.</i>
30-4-36 ..	(4) 2	10,320
1-5-36 ..	(5) 2	9,760	39·0	3,809	1·92	54·0	5,270	<i>Nil.</i>
2-5-36 ..	(6) 2	8,660
4-5-36 ..	(7) 2	7,500	46·5	3,487	2·09	47·5	3,562	<i>Nil.</i>
5-5-36 ..	(8) 2	9,600
6-5-36 ..	(9) 2	11,200	53·5	5,992	2·02	40·0	4,490	<i>Nil.</i>
7-5-36 ..	(10) 2	11,000
8-5-36 ..	(11) 2
9-5-36 ..	(12) 2	8,000	49·0	3,920	1·98	45·0	3,600	<i>Nil.</i>
11-5-36 ..	(13) 2	7,640	45·5	3,476	2·04	48·5	3,705	Normo 1/300.
12-5-36 ..	(14) 2
13-5-36	8,640	40·5	3,509	1·89	54·5	4,709	Normo 1/300.

Comment.—It is evident from my experiments on rabbits and from the experiments of Powers and Murphy (*loc. cit.*), Meyer, Middleton and Thewlis (*loc. cit.*), and Powers (*loc. cit.*) on human beings that single injection of liver extract in large doses produces a leucocytosis of short duration due to an increase in the neutrophils. The leucocytosis is observed three to four hours after an intravenous injection and six to seven hours after an intramuscular injection. Evidently the leucocytosis is not merely due to re-distribution of cells from the viscera but to a certain extent to influx of pre-formed cells from the bone-marrow, as is evident from an increase of staff forms and consequent lowering of the weighted mean. That the leucocytosis was not due to squeezing of the spleen was almost conclusively proved by Meyer and others who demonstrated leucocytosis with the injection of the drug in one case of Banti's disease and in another case of hæmolytic icterus, both before and after

splenectomy. It is nevertheless difficult to agree with Powers (*loc. cit.*) in his assertion that the leucocytosis was due to direct or indirect stimulation of the marrow. If the leucocytosis was due to stimulation of the marrow, repeated injections of the drug should have caused a sustained leucocytosis if the marrow was not completely devoid of leucopoietic cells, but no leucocytosis was maintained, as will be seen from the chart, though the marrow was slightly hyperplastic. If the hyperplasia of the marrow was due to the stimulating effect of the drug, that stimulation was not sufficient to induce a sustained leucocytosis in the peripheral blood. The leucocytosis observed within three to seven hours after the injection of the drug is probably due to some transient chemotactic action by which pre-formed reserve cells from the marrow are temporarily drawn into the peripheral circulation, but after only a short stay the cells leave to stagnate in the various viscera of the body.

3. COLCHICINE.

Colchicine exerts two distinct types of action—one immediate, like most alkaloids, and the other remote, after it has been in the circulation for some hours, like the toxins. The immediate effects are exerted on plain muscle and the automatic movements of the plain muscle throughout the body are augmented. Thus, peristalsis is increased and splenic, uterine, and bronchiolar muscles show augmented tonus and movements. The remote effect comes on very slowly and is shown as gradual paralysis of the central nervous system, bearing a close resemblance to the effects of snake poison and toxins.

Dixon and Malden (1908) made thorough studies of the pharmacological action of colchicine with special reference to its action on the blood. Their studies on the blood were based on elaborate experiments on rabbits, rats, dogs, and in a few cases on men. The drug was administered in watery solution of 0.5 to 1 per cent by subcutaneous injection. They found that after the injection of a single large dose, 0.005 g. to 0.02 g., of colchicine in rabbits, there is at first a stage of leucopenia which is seen one hour after the injection and is almost entirely due to neutropenia, the decrease of the lymphocytes being relatively insignificant. The white cells return to normal levels three hours after injection, mainly due to increase in lymphocytes. The white cell count continues to increase and attains its maximum level 18 to 24 hours after the injection. The increase up to the eighth and tenth hours is mainly due to lymphocytosis, after which the lymphocytes fall to the normal level, and the increase at the maximum level at 18 to 24 hours is mainly due to increase in the neutrophils. Immature cells, myelocytes as well as normoblasts, were frequently seen at the later stages of the experiment. Similar experiments conducted by the same authors on rats, dogs, and on men showed the same type of action on the blood.

My experiments on the action of colchicine on the blood of rabbits corroborated exactly the results obtained by Dixon and Malden (Chart 5, Table V). The initial leucopenia was mainly due to a fall in neutrophils. The subsequent rise in leucocytes was due to increase in the lymphocytes up to the eighth hour but the leucocytosis at the 24th hour was due entirely to the increase in the neutrophils, the lymphocytes having regained the level before injection at the 48th hour. Myelocytes and metamyelocytes were found in large numbers from the

fifth to 24th hour; an occasional normoblast was also found during this period. Additional evidence of the presence of young white cells was found in the lowering of the weighted mean due to increase in the staff neutrophils. The 'weighted mean' continued to be low even at the 48th hour, when the leucocytes had reached their normal level, but it came back to the original pre-injection level on the third day after the injection.

CHART 5.

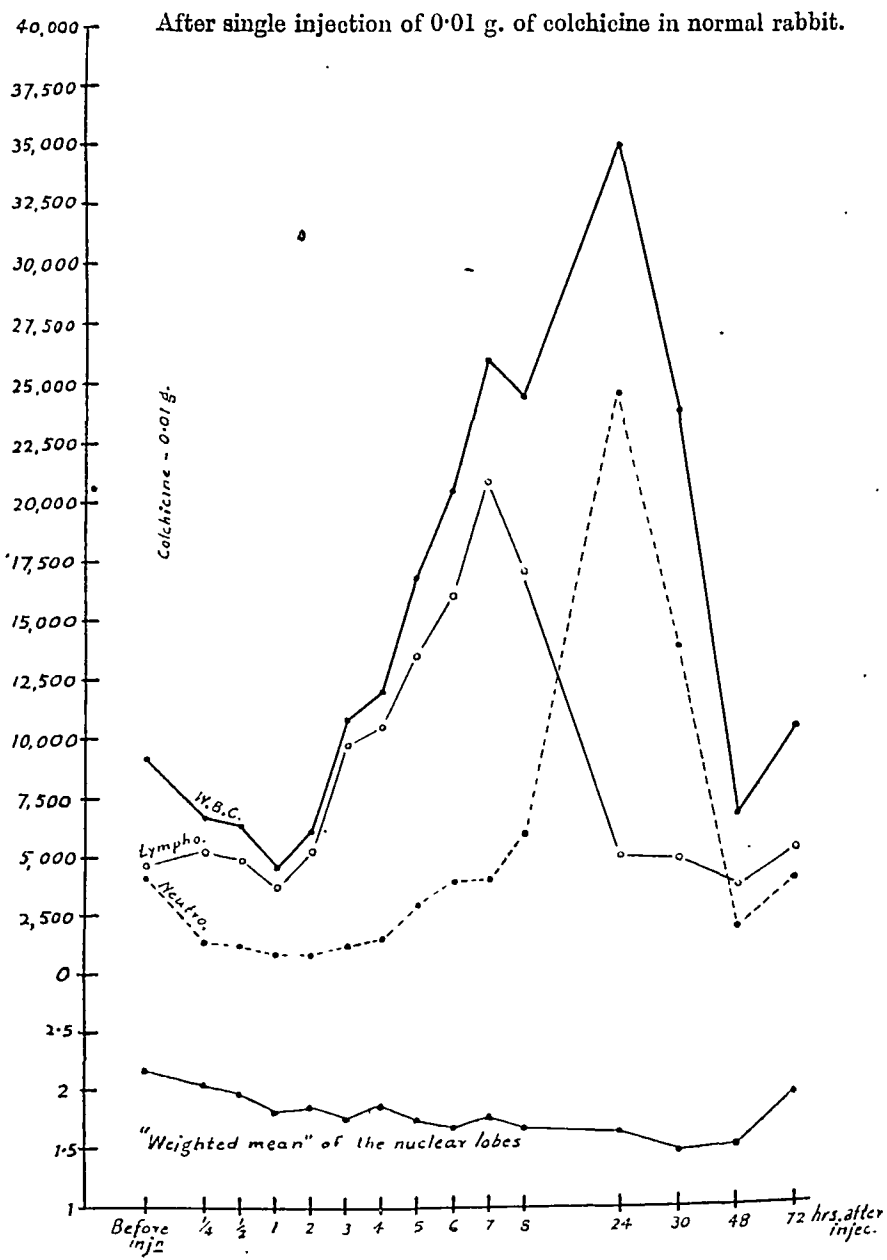


TABLE V.

After single subcutaneous injection of 0.01 g. of colchicine.

Time.	W. B. C. total.	NEUTROPHILS.		'Weighted mean.'	LYMPHOCYTE.		Immature cell, per cent.
		Per cent.	Total.		Per cent.	Total.	
Before injection	9,200	44.0	4,018	2.17	50.0	4,600	Nil.
$\frac{1}{4}$ hour after injection	6,640	20.0	1,328	2.03	76.5	5,080	Nil.
$\frac{1}{2}$ " " "	6,300	18.5	1,124	1.96	78.0	4,914	Nil.
1 " " "	4,500	18.0	810	1.80	80.0	3,600	Nil.
2 hours " "	6,000	13.0	780	1.84	84.0	5,040	Nil.
3 " " "	10,660	10.0	1,066	1.74	90.0	9,594	Normo 1/200.
4 " " "	11,900	12.0	1,428	1.86	87.0	10,353	Normo 1/200.
5 " " "	16,600	17.0	2,832	1.72	82.0	13,661	Met 2, Normo 1/200.
6 " " "	20,280	19.0	3,853	1.68	78.0	15,818	Met 1.7, Normo 1/200.
7 " " "	25,720	15.5	3,987	1.74	80.0	20,576	Met 4, Normo 3/200.
8 " " "	24,340	24.0	5,842	1.66	69.5	16,916	Normo 2/200, Met 5, Mmet 1.5.
24 " " "	34,920	70.0	24,444	1.63	14.5	4,943	Normo 1/200, Met 6.5, Mmet 7.5.
30 " " "	23,560	58.0	13,665	1.48	20.5	4,830	Normo 1/200, Met 8, Mmet 7.
48 " " "	6,660	30.0	1,998	1.50	53.0	3,530	Met 6, Mmet 5.
72 " " "	10,200	39.0	3,978	1.93	50.0	5,010	Normo 1/200, Met. 1.

Met = Myelocyte.

Mmet = Metamyelocyte.

Dixon and Malden found that when the animal was killed in the stage of initial leucopenia, one hour after injection, the marrow was teeming with cells, while in another animal, when the section was made 12 hours after a single dose of colchicine at a time when there was marked leucocytosis in the peripheral circulation, the marrow was almost denuded of cells. Sections made from normal animals from the same litter showed a condition as regards the number of cells intermediate between the above two. Almost similar results were observed by me in the bone-marrow of the rabbits. In a rabbit which was killed four hours after a dose of 0.0075 gramme of colchicine subcutaneously, the leucocytes were going up and had already risen from 6,200 to 10,000 per c.mm. in the peripheral circulation. There was preponderance of leucocytes throughout the marrow smear, there being as many as over 30 per cent neutrophil myelocytes; the section was also very cellular, leucopoietic areas being far in excess of erythropoietic areas and the whole section teeming with granular cells. In another rabbit which was killed 16 hours after the injection of 0.01 gramme of colchicine, when the peripheral leucocytes had gone up from 10,120 to 20,000 per c.mm., cells were scanty in the marrow smear with a preponderance of erythroblastic cells; 54 per cent of the total cells were normoblasts, and the section was denuded of marrow cells except in patches where erythroblastic activity was far in excess of leucoblastic.

Action on splenectomized rabbits.—When the drug was administered in the same dose to a splenectomized rabbit, the effect on the blood (Chart 5-B, Table V-b) was about the same with the exception that the leucocytosis due to the increase of the lymphocytes did not manifest itself till the sixth hour.

Effect of small repeated doses of colchicine.—Dixon and Malden gave small doses of colchicine at irregular intervals over several weeks. The animals showed no ill effects at any time, the leucocytes remained almost steady and the ratio of polymorphs to lymphocytes also remained constant throughout the experiment. Abnormal cells were occasionally found, a myelocyte and a few normoblasts now and then. At the end of the experiment when the animals were killed certain changes were evident in the bone-marrow. The marrow cells increased in number, while the fat cells tended to disappear. The cells showed plentiful mitotic figures and appeared to be undergoing multiplication.

Exactly similar results were obtained in a rabbit (Chart 5-A, Table V-a) though the dose was higher than given by Dixon and Malden and injections were made daily except Sundays up to 21 injections in all. The animal appeared in good health during the period of the experiment and even gained in weight. Fluctuations of the total and differential count were within the normal range of variation except that in the later period of the experiment persistent neutropenia was noticed. The 'weighted mean' began to fall after the fourth injection and a low level was maintained up to the end. A few normoblasts were almost always present after the eighth injection, while myelocytes were found almost invariably after the thirteenth injection. Toxic polymorphs, small cells with coarse acidophilic granules, were always found after the sixth injection. Hæmoglobin fell from initial 69 per cent to 56 per cent and total red blood cells fell from initial count of 5,160,000 to 3,940,000 per c.mm. at the end of the experiment, while the reticulocyte percentage remained almost constant throughout.

CHART 5-A.

After repeated injections of colchicine.

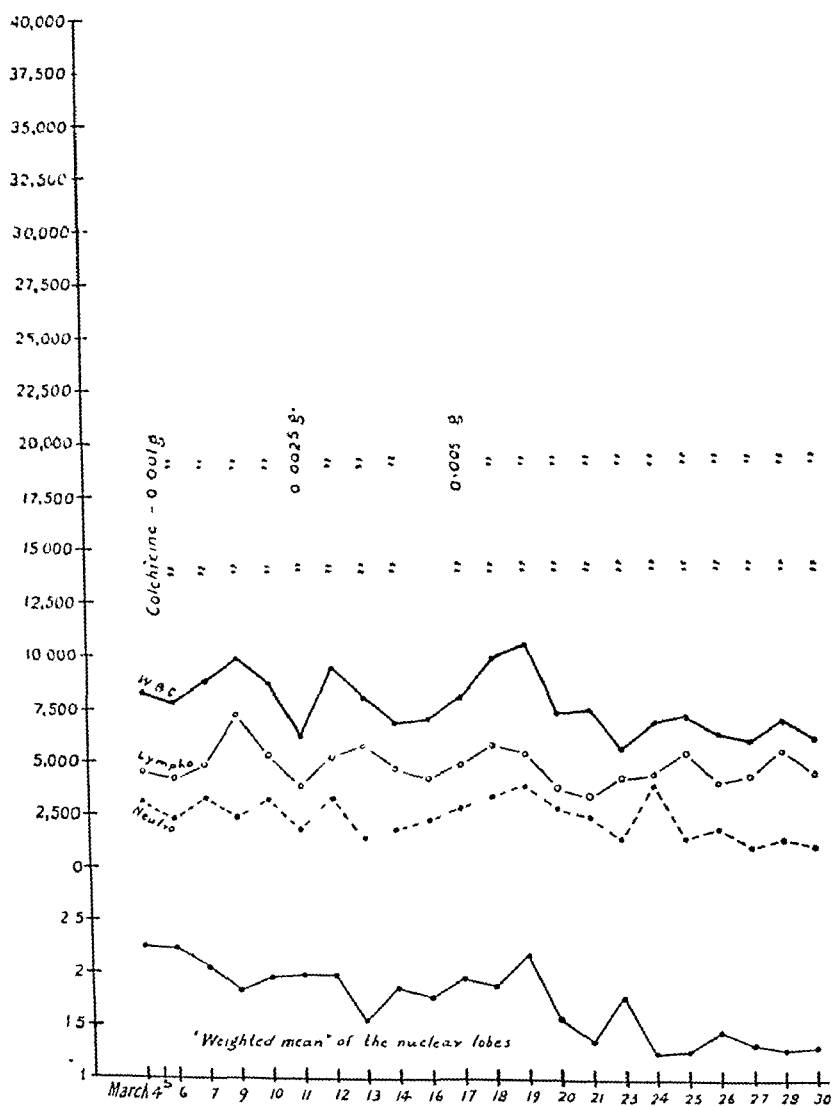


CHART 5-B.

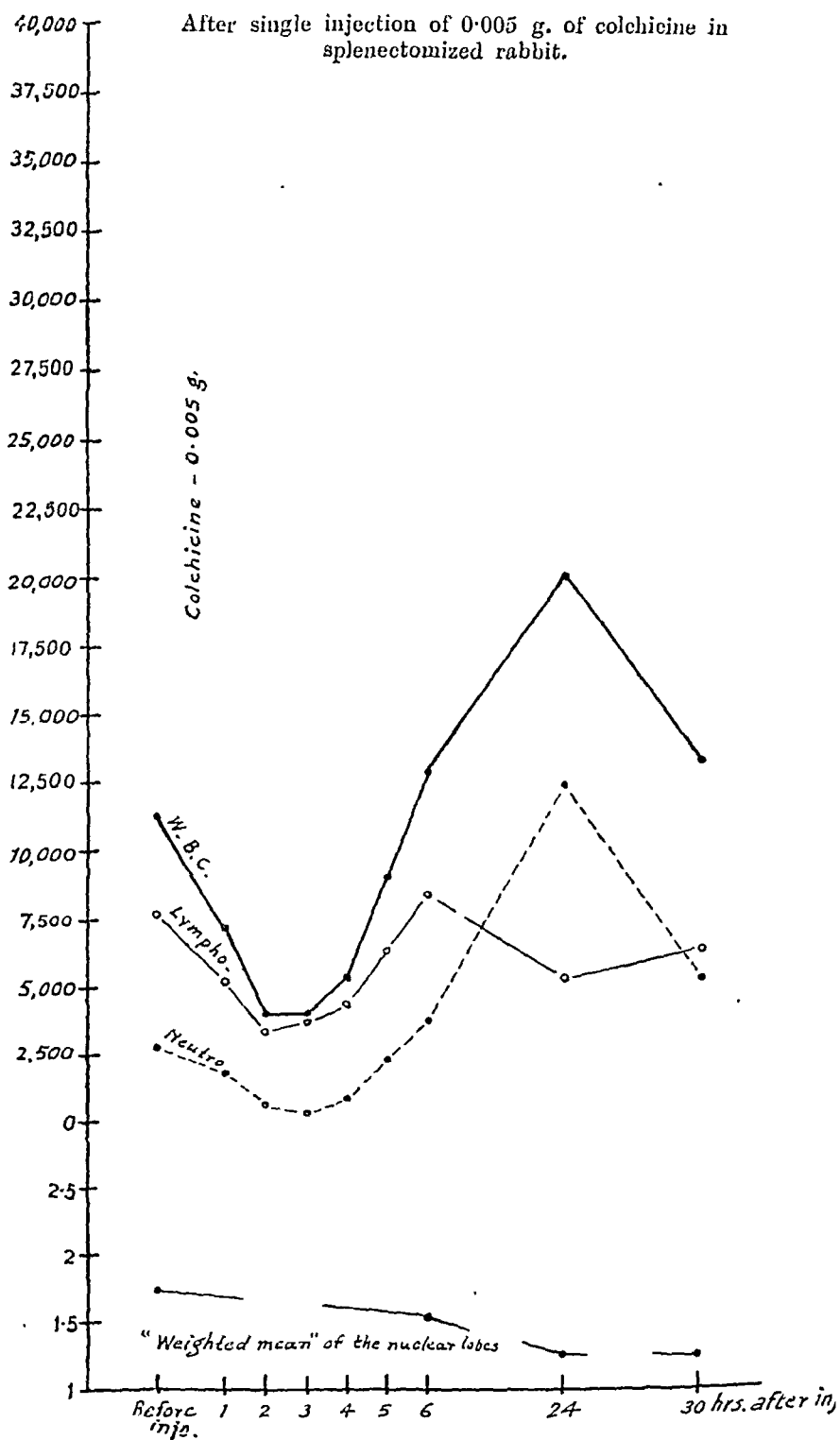


TABLE V-a.

After repeated subcutaneous injections of colchicine.

Date.	Number and amount, g.	W. B. C. total.	NEUTROPHILS.		'Weighted mean.'	LYMPHOCYTE.		Immature cell, per cent.
			Per cent.	Total.		Per cent.	Total.	
4-3-36	8,400	37.0	3,108	2.27	54.0	4,536	Nil.
5-3-36 ..	(1) 0.001
6-3-36 ..	(2) 0.001	7,800	31.0	2,418	2.24	56.0	4,368	Nil.
7-3-36 ..	(3) 0.001	8,860	37.0	3,278	2.05	56.0	4,951	Nil.
9-3-36 ..	(4) 0.001	9,800	25.0	2,450	1.82	73.0	7,158	Nil.
10-3-36 ..	(5) 0.001	8,640	35.0	3,024	1.96	61.0	5,270	Met 0.3.
11-3-36 ..	(6) 0.0025	6,140	28.0	1,719	1.98	64.0	3,929	Nil.
12-3-36 ..	(7) 0.0025	9,400	34.0	3,196	1.98	54.0	5,076	Nil.
13-3-36 ..	(8) 0.0025	8,000	17.0	1,360	1.54	71.0	5,680	Nil.
14-3-36 ..	(9) 0.0025	6,900	24.0	1,656	1.86	66.0	4,554	Normo 3/200.
16-3-36 ..	(10) 0.0025	7,000	31.0	2,170	1.78	59.0	4,130	Normo 1/200.
17-3-36 ..	(11) 0.005	8,000	34.0	2,720	1.92	61.0	4,800	Normo 2/200.
18-3-36 ..	(12) 0.005	9,740	35.0	3,409	1.89	58.0	5,649	Normo 3/200.
19-3-36 ..	(13) 0.005	10,320	36.0	3,775	2.14	55.0	5,165	Nil.
20-3-36 ..	(14) 0.005	7,100	36.5	2,590	1.56	51.5	3,662	Met 0.3.
21-3-36 ..	(15) 0.005	7,400	34.0	2,416	1.34	44.0	3,256	Met 4.0.
23-3-36 ..	(16) 0.005	5,500	21.0	1,155	1.74	73.0	4,015	Normo 1/200.
24-3-36 ..	(17) 0.005	9,200	41.0	3,772	1.21	47.0	4,324	Met 3, 1/200.
25-3-36 ..	(18) 0.005	7,000	17.0	1,190	1.24	74.0	5,180	Met 1, 3/200.
26-3-36 ..	(19) 0.005	6,140	25.0	1,535	1.42	65.0	3,991	Met 1, 1/200.
27-3-36 ..	(20) 0.005	5,400	17.0	918	1.30	76.0	4,104	Met 1, 1/200.
28-3-36 ..	(21) 0.005	6,800	15.0	1,020	1.28	73.0	5,436	Met 3.
30-3-36	6,000	16.0	960	1.30	74.0	4,440	Met 2, 2/200.

Met = Myelocyte.

CHART 6.

After single injection of 1 c.c. of adrenaline chloride (P. D.) in normal rabbit.

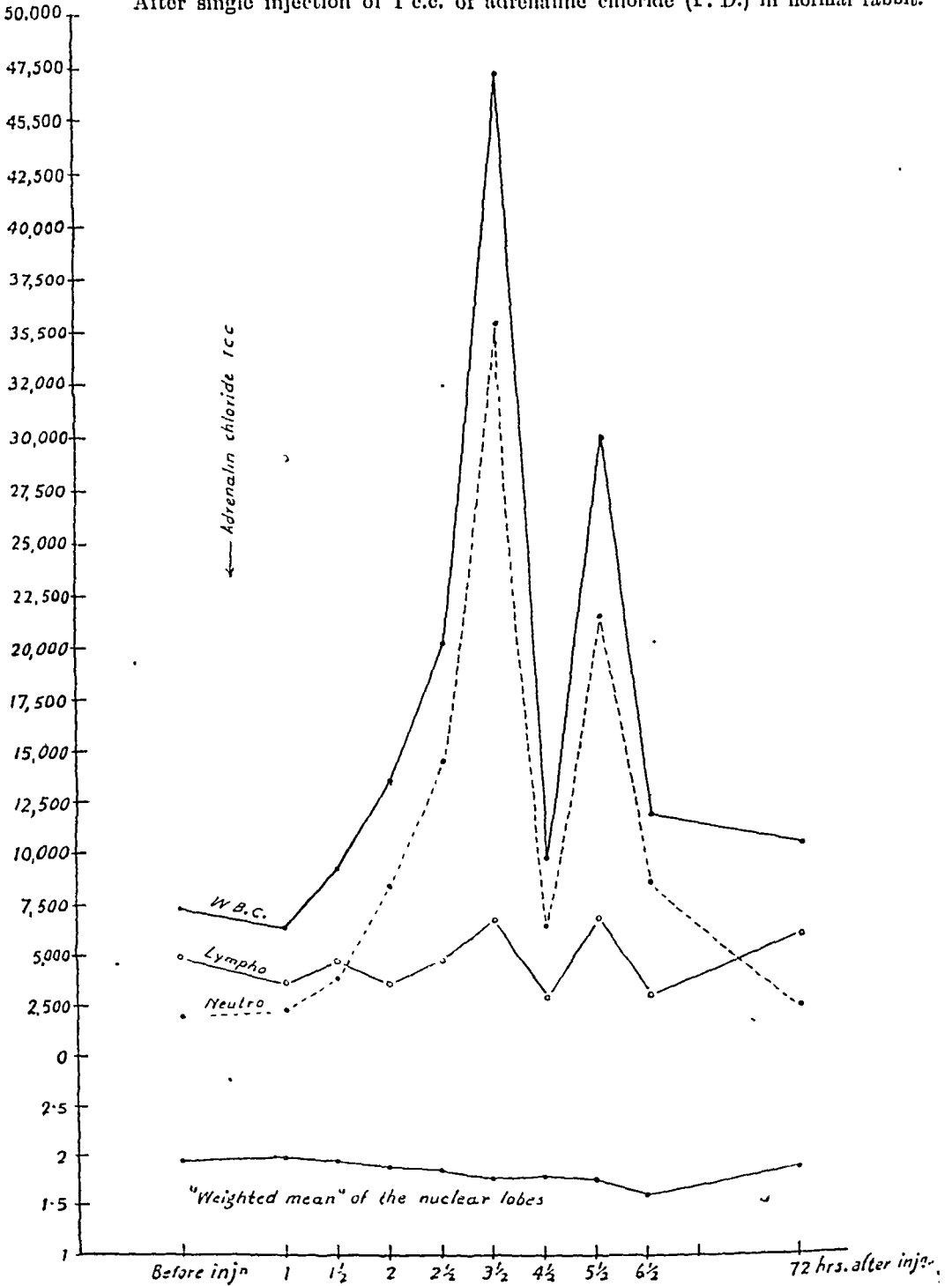


TABLE VI.

After single injection of 1 c.c. of adrenaline chloride in a normal rabbit.

Time.	W. B. C. total.	NEUTROPHILS.		'Weighted mean.'	LYMPHOCYTE.		Immature cell, per cent.
		Per cent.	Total.		Per cent.	Total.	
Before injection ..	7,320	27.0	1,976	1.94	68.0	4,978	Nil.
½ hour after injection ..	8,000
1 " " " ..	6,400	35.0	2,240	1.96	55.0	3,520	Nil.
1½ hours " " ..	9,120	42.0	3,830	1.92	50.0	4,560	Nil.
2 " " " ..	13,460	62.0	8,345	1.88	26.0	3,500	Nil.
2½ " " " ..	20,000	72.0	14,400	1.83	23.0	4,600	Nil.
3½ " " " ..	47,100	75.0	35,325	1.76	14.0	6,594	Nil.
4½ " " " ..	9,540	67.0	6,392	1.79	30.0	2,862	Nil.
5½ " " " ..	20,800	73.0	21,228	1.76	23.0	6,688	Normo 1/300.
6½ " " " ..	11,600	73.0	8,468	1.60	25.0	2,900	Nil.
72 " " " ..	10,120	27.0	2,311	1.84	68.0	5,821	Nil.

noticed along with the increase in neutrophils. The rise in leucocytes was very marked in two rabbits but in the third it reached a maximum level of only 13,000

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from the original 6,000. When 1 c.c. of adrenaline chloride was injected into a splenectomized rabbit—the same course of events was noticed as in the healthy animal, viz., an initial rise at one and a half hours, then a fall and then a secondary rise again at four and a half hours after the injection (Chart 6-A,

CHART 6-A.

After single injection of 1 c.c. of adrenaline chloride in splenectomized rabbit.

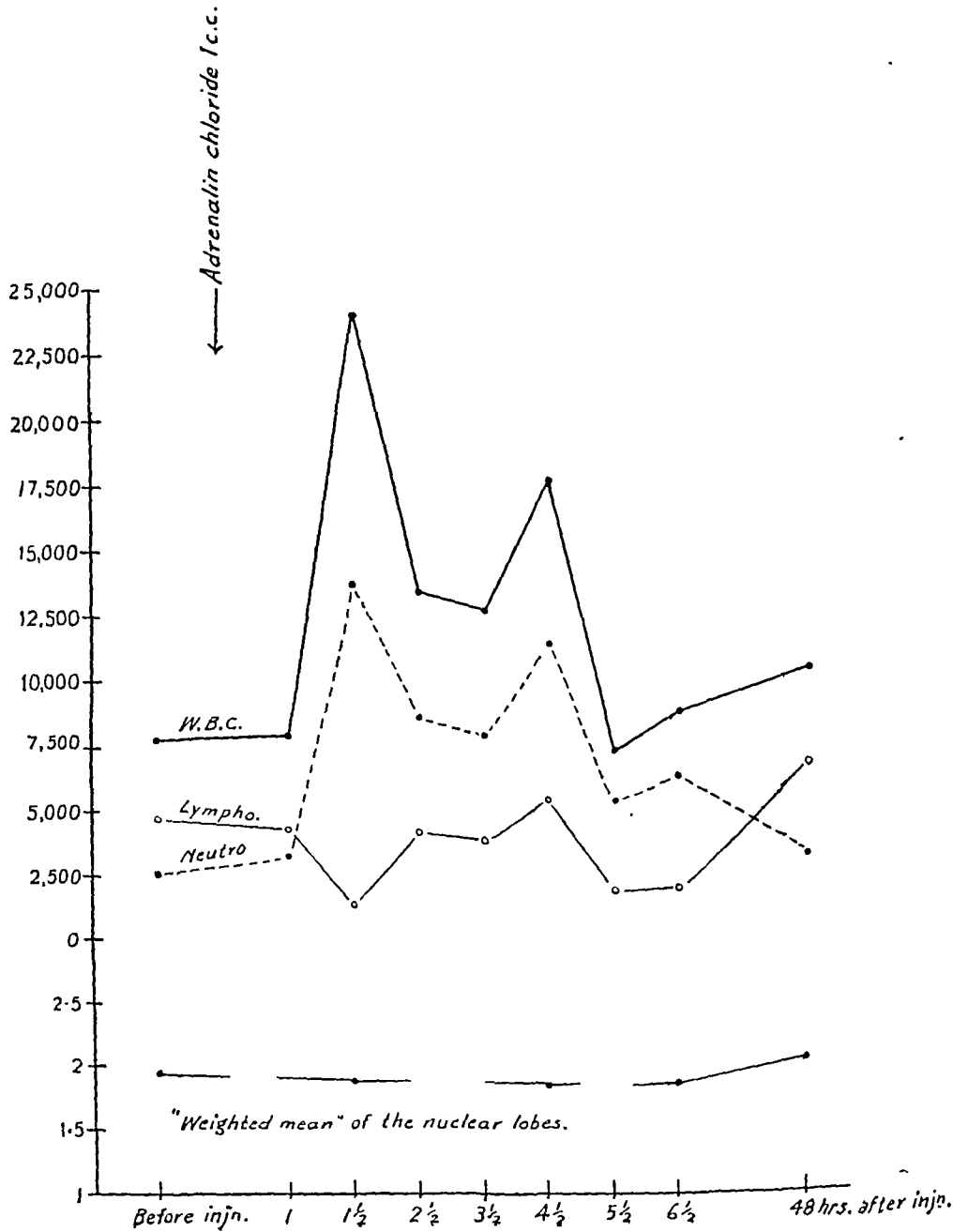


Table VI-a). Here too the initial rise was due to an increase in neutrophils and lymphocytes—the increase in neutrophils being proportionately much greater than that of the lymphocytes, but the subsequent rise was entirely due to increase in the neutrophils. No immature white cell was found at any time in any of the cases and the ‘weighted mean’ in the splenectomized animal remained almost constant throughout the experiments, though it was slightly lowered in the normal animals.

TABLE VI-a.

After single injection of 1 c.c. of adrenaline chloride in a splenectomized rabbit.

Time.	W. B. C. total.	NEUTROPHILS.		‘Weighted mean.’	LYMPHOCYTE.		Immature cell, per cent.
		Per cent.	Total.		Per cent.	Total.	
Before injection ..	7,760	33.0	2,561	1.96	61.0	4,734	Nil.
1 hour after injection ..	7,920	41.0	3,247	..	55.0	4,356	Nil.
1½ hours “ “ ..	24,000	49.0	13,760	1.80	43.0	1,320	Nil.
2½ “ “ “ “ ..	13,400	64.0	8,566	..	31.0	4,154	Normo 1/300.
3½ “ “ “ “ ..	12,600	63.0	7,938	..	31.5	3,969	Nil.
4½ “ “ “ “ ..	17,600	65.0	11,440	1.84	31.0	5,456	Nil.
5½ “ “ “ “ ..	7,400	72.0	5,328	..	26.0	1,924	Nil.
6½ “ “ “ “ ..	8,800	73.5	6,468	1.86	26.0	1,936	Nil.
48 “ “ “ “ ..	10,400	31.0	3,224	2.01	66.0	6,864	Nil.

Comment.—It has been conclusively shown that injected subcutaneously adrenaline chloride produces a sharp leucocytosis in normals, in splenectomized men and animals, and in patients with enlarged spleens. But whereas the leucocytosis observed by Patek and Daland (*loc. cit.*) was due to increase in both myeloid and lymphoid cells, the leucocytosis observed by me was almost entirely due to increase in the neutrophils, except in the case of a splenectomized rabbit where an increase in the lymphocytes was noticed as well in the early stage. Though it is universally accepted from experimental work that adrenaline chloride causes a strong contraction of the spleen and though Barcroft's suggestion that the spleen may serve as reservoir for the circulating blood is also accepted, it is quite evident that the leucocytosis observed after injection of adrenaline chloride does not originate from the spleen. The absence of any immature cells at any period in most cases negatives the idea of stimulation of leucopoiesis. Patek and Daland prefer to explain the increase in the white cells by a mechanical alteration in the blood stream. Cells either pooled in inactive vascular beds or lying along the periphery of vessel walls may be thrust into the axial blood stream by active contraction of the blood vessels or by increased velocity of the axial flow. I fully agree with Patek and Daland that the initial leucocytosis is caused by mobilization of cells which have stagnated in the viscera as a result of active contraction of blood vessels. But this action is not sufficient to explain the prolonged leucocytosis observed by Patek and Daland or the second leucocytosis about three to four hours after injection observed by me. The late leucocytosis appears to be due, at least to a certain degree, to mobilization of pre-formed reserves from the marrow, as is shown by the slight lowering of the 'weighted mean' during that period.

5. HISTAMINE.

Most of the earlier observations on the action of histamine on animals and human beings were incidental to the use of the drug for therapeutic and other purposes and in most cases the data were fragmentary and even contradictory. Recently, Moon *et al.* (1935) made some studies on the action of histamine on cats, monkeys, and on human volunteers. In every instance they found that the injection of histamine was followed by leucocytosis due to an increase in the number of polymorphonuclear leucocytes. The maximum leucocytosis occurred in about three to five hours, and the degree of leucocytosis was not so marked in men as in animals. Frequently the leucocytosis was preceded by a transient period of leucopenia. The count generally returned to the normal level in about 24 hours.

The effect of histamine was noted in two normal and one splenectomized rabbit, 0.5 milligram of histamine being injected in one normal, while 1.5 milligrams of the drug was injected in the other two animals. The animals tolerated the drug very well and did not show any sign of shock. Leucocytosis due to increase in the neutrophils was seen in all three, the maximum increase being between two and four hours. A stage of initial leucopenia was not seen in any of the three animals though the blood was examined at intervals of 15 minutes for the first hour. Slight increase in the neutrophils was responsible for the lowering of the weighted mean in the two normal animals but no immature cell was found at any time in any of the animals. The count came back to original pre-injection level after 24 hours (Charts 7, 7-A, Tables VII, VII-a).

CHART 7.

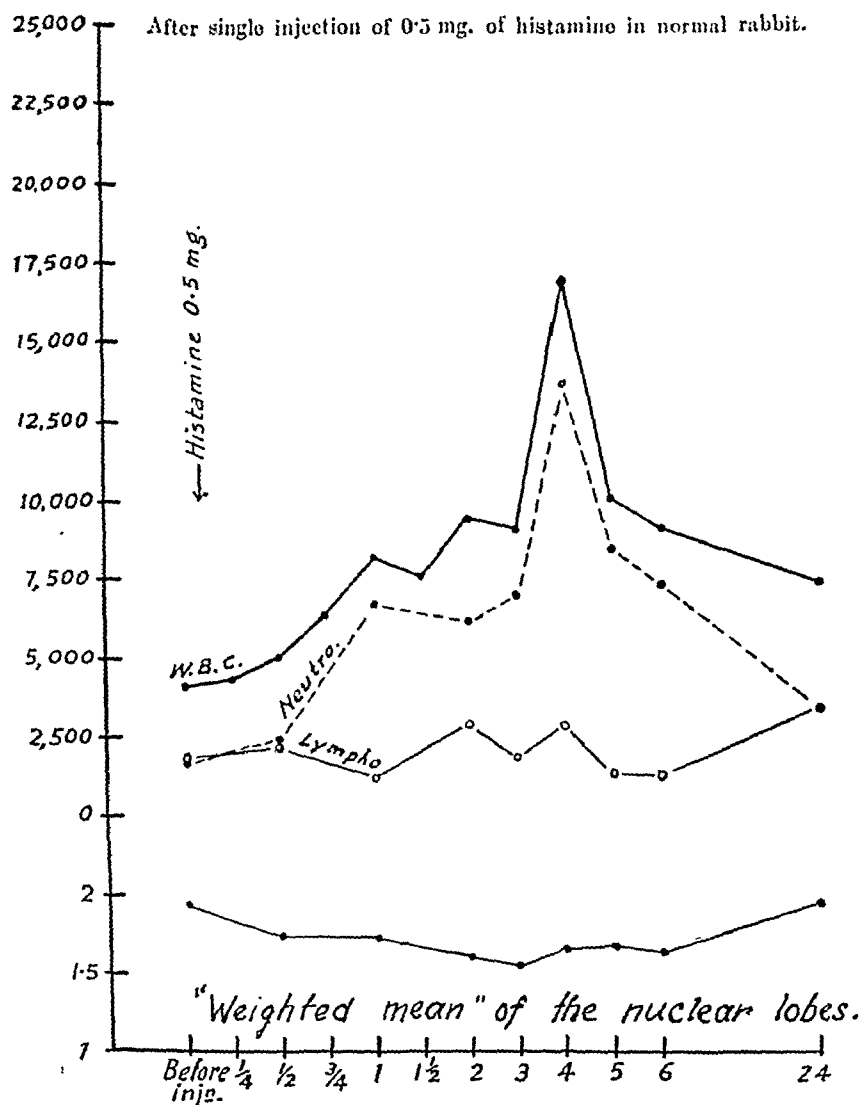


CHART 7-A.

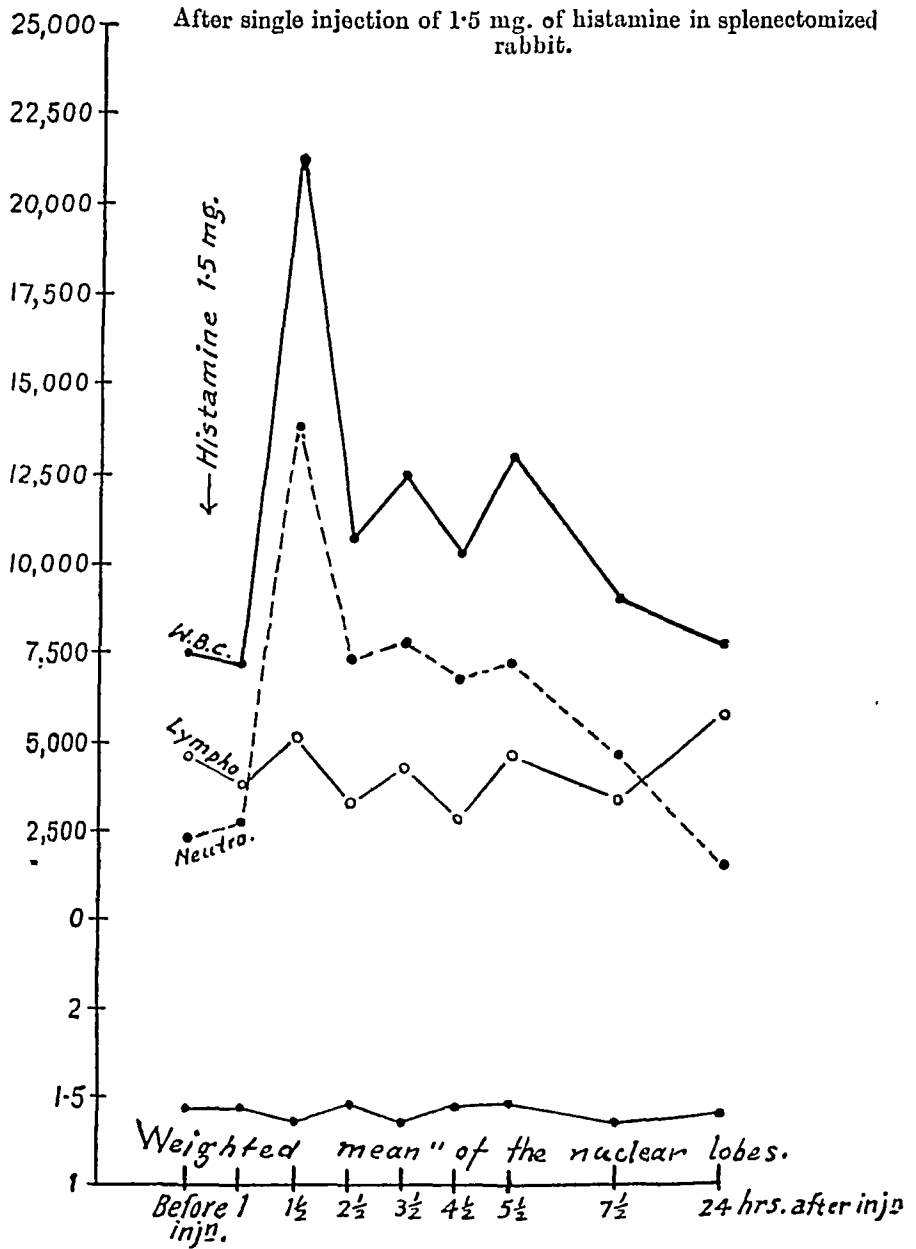


TABLE VII.

After single injection of 0.5 mg. of histamine in a normal rabbit.

Time.	W. B. C. total.	NEUTROPHILS.		'Weighted mean.'	LYMPHOCYTE.		Immature cell, per cent.
		Per cent.	Total.		Per cent.	Total.	
Before injection ..	4,080	46.0	1,877	1.94	48.0	1,958	<i>Nil.</i>
$\frac{1}{4}$ hour after injection ..	4,350
$\frac{1}{2}$ " " " ..	5,000	49.0	2,450	1.74	44.0	2,200	<i>Nil.</i>
$\frac{3}{4}$ " " " ..	6,400
1 " " " ..	8,080	82.0	6,626	1.72	15.5	1,252	<i>Nil.</i>
$1\frac{1}{2}$ hours " " ..	7,500
2 " " " ..	9,400	64.0	6,016	1.60	31.5	2,961	<i>Nil.</i>
3 " " " ..	9,000	77.0	6,930	1.55	20.0	1,800	<i>Nil.</i>
4 " " " ..	16,800	80.5	13,534	1.64	17.0	2,856	<i>Nil.</i>
5 " " " ..	10,000	84.0	8,400	1.68	14.0	1,400	<i>Nil.</i>
6 " " " ..	9,000	81.0	7,290	1.62	14.0	1,260	<i>Nil.</i>
$2\frac{1}{4}$ " " " ..	7,340	45.0	3,303	1.92	45.0	3,303	<i>Nil.</i>

The Action of Leucopoietic Drugs.

TABLE VII-a.

After single subcutaneous injection of 1.5 mg. of histamine in a splenectomized rabbit.

Time.	W. B. C. total.	NEUTROPHILS.		'Weighted mean.'	LYMPHOCYTE.		Immature cell, per cent.
		Per cent.	Total.		Per cent.	Total.	
Before injection ..	7,500	32.0	2,400	1.42	61.5	4,613	<i>Nil.</i>
1 hour after injection ..	7,200	37.5	2,700	1.42	54.0	3,888	<i>Nil.</i>
1½ hours " " ..	21,200	66.0	13,992	1.38	24.0	5,088	<i>Nil.</i>
2½ " " " ..	10,800	68.0	7,344	1.46	31.0	3,348	<i>Nil.</i>
3½ " " " ..	12,500	61.0	7,625	1.36	34.0	4,250	<i>Nil.</i>
4½ " " " ..	10,400	66.0	6,824	1.44	28.0	2,912	<i>Nil.</i>
5½ " " " ..	13,000	56.0	7,280	1.47	36.0	4,680	<i>Nil.</i>
7½ " " " ..	9,000	51.5	4,635	1.37	38.5	3,465	<i>Nil.</i>
24 " " " ..	7,600	21.0	1,596	1.40	76.0	5,776	<i>Nil.</i>

Comment.—It has been almost conclusively proved from experiments on animals and men that injection of moderately high doses of histamine is followed by a considerable leucocytosis. The leucocytosis reaches the highest peak in two to four hours and is due entirely to increase in the neutrophils. In most cases, the work of Moon and his colleagues not being excepted, data are rather meagre and do not allow of

full critical examination as to the source of the leucocytosis. But Flatow and Huttel in their experiments found some immature forms and also noted a shift to the left of the Arneht count denoting the participation of the marrow in the leucocytosis. A lowering of the 'weighted mean' due to an increased number of staff neutrophils was also observed by me though no immature cell was seen in any of the cases. From the fact that the leucocytosis was entirely due to the neutrophils as well as from an experiment on a splenectomized rabbit where the usual neutrophilic leucocytosis was seen after an injection of histamine, it is reasonable to conclude that the source of leucocytes could not be the spleen. From the evidence to hand, the leucocytosis is probably due to mobilization of pre-formed reserves from the marrow. The stage of initial leucopenia noted by some observers was probably due to a comparatively large dose of the drug, as will be evident from the transient leucopenia which is observed following a shock dose of histamine in rabbits and the marked leucopenia accompanying shock which was obtained by Dale and Laidlaw (1919).

6. ACETYL-CHOLINE AND CARBAMINOYL-CHOLINE.

Only a brief reference was found to the action of acetyl-choline on the blood or blood-forming organs, Backman *et al.* (1925) stating that acetyl-choline increases the number of platelets but does not modify that of leucocytes. I, therefore, studied the action of acetyl-choline and carbaminoyl-choline on five rabbits. Two normal rabbits received injection of acetyl-choline, one 0.025 gramme and the other 0.0187 gramme. One splenectomized rabbit received 0.025 gramme of the same drug, while two normal rabbits received 0.000125 gramme of carbaminoyl-choline. The injections were given intramuscularly and all the animals tolerated the injections very well and did not show any toxic effect. All the animals—four normal and one splenectomized—showed almost identical blood pictures with both drugs. An initial leucocytosis about one to one and a half hours after the injection was followed by leucopenia which was succeeded by another rise in the total white cell count in about four to six hours; finally the count settled down to the original level before injection in about 24 hours. The height of the initial leucocytosis was greater in normal animals after acetyl-choline, while the height of the final leucocytosis was greater in the two normal rabbits after carbaminoyl-choline and in the one splenectomized rabbit after acetyl-choline. In all the animals the initial leucocytosis was due to an increase in both neutrophils and lymphocytes, the rise in neutrophils being always greater than that of the lymphocytes, while the final leucocytosis was due entirely to increase in the neutrophils. In the splenectomized animal, the increase in the neutrophils alone was responsible for both initial and late leucocytosis. The leucopenia was due to a fall both in neutrophils and lymphocytes after acetyl-choline, whereas after carbaminoyl-choline it was almost entirely due to a fall in lymphocytes, the level of neutrophils still continuing at a higher point than before the injection. A few normoblasts were seen in one animal after acetyl-choline, while a myelocyte was seen in one animal after carbaminoyl-choline. There was an increase in staff neutrophils in all cases during the period of late leucocytosis, manifested in lowering of the 'weighted mean'.

CHART 8.

After single injection of 0.025 g. of acetyl-choline in normal rabbit.

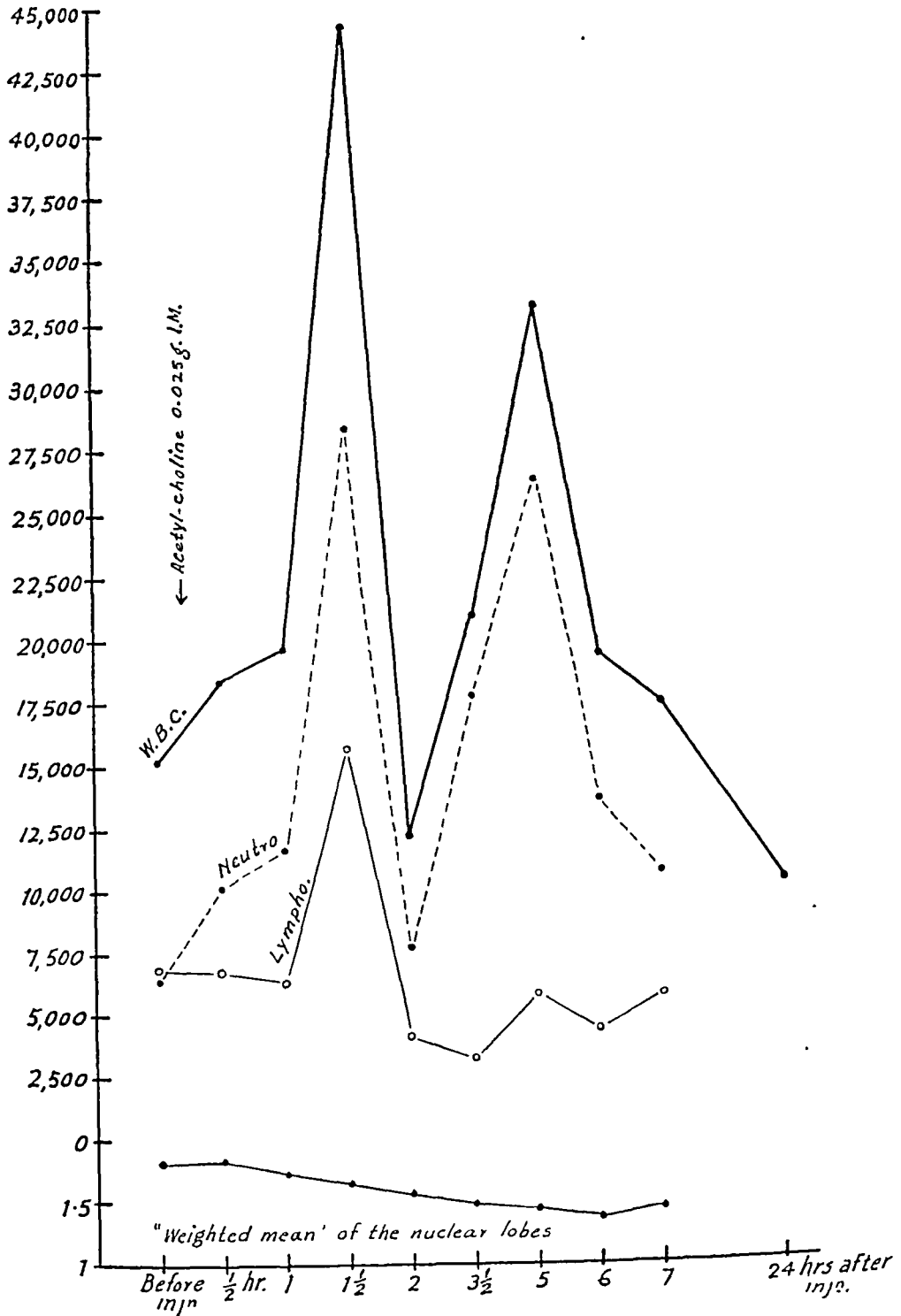


CHART 8-A.

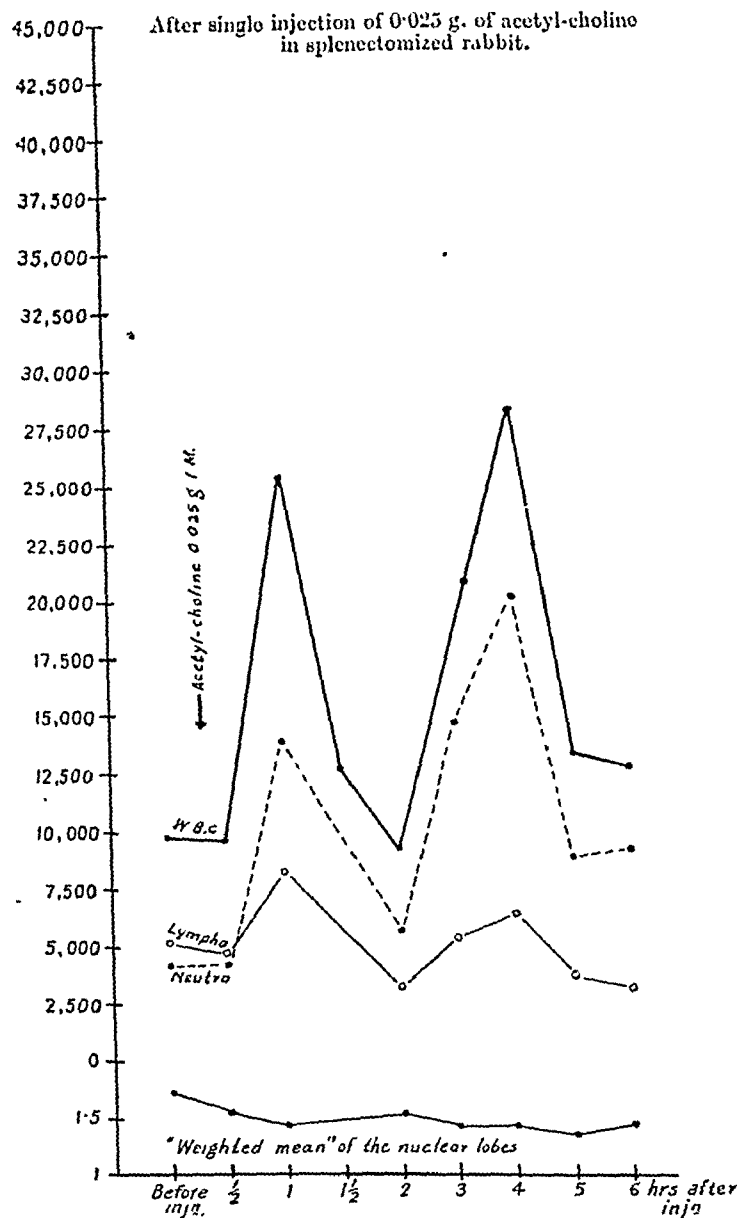


TABLE VIII.

After single subcutaneous injection of 0.025 g. of acetyl-choline in a normal rabbit.

Time.	W. B. C. total.	NEUTROPHILS.		'Weighted mean.'	LYMPHOCYTE.		Immature cell, per cent.
		Per cent.	Total.		Per cent.	Total.	
Before injection ..	15,100	43.0	6,490	1.81	46.0	6,940	<i>Nil.</i>
$\frac{1}{2}$ hour after injection ..	18,300	53.0	10,065	1.82	37.0	6,771	<i>Nil.</i>
1 " " " ..	19,600	60.0	11,507	1.72	32.5	6,390	<i>Nil.</i>
1 $\frac{1}{2}$ hours " " ..	44,350	63.0	27,940	1.62	35.0	15,518	<i>Nil.</i>
2 " " " ..	12,200	63.0	7,686	1.58	33.0	4,026	<i>Nil.</i>
3 $\frac{1}{2}$ " " " ..	20,960	84.0	17,606	1.49	15.0	3,144	<i>Nil.</i>
5 " " " ..	33,120	79.5	26,302	1.42	17.5	5,796	<i>Nil.</i>
6 " " " ..	19,400	70.0	13,580	1.37	23.0	4,460	<i>Nil.</i>
7 " " " ..	17,480	61.0	10,662	1.42	33.0	5,768	<i>Nil.</i>
24 " " " ..	10,280

TABLE VIII-a.

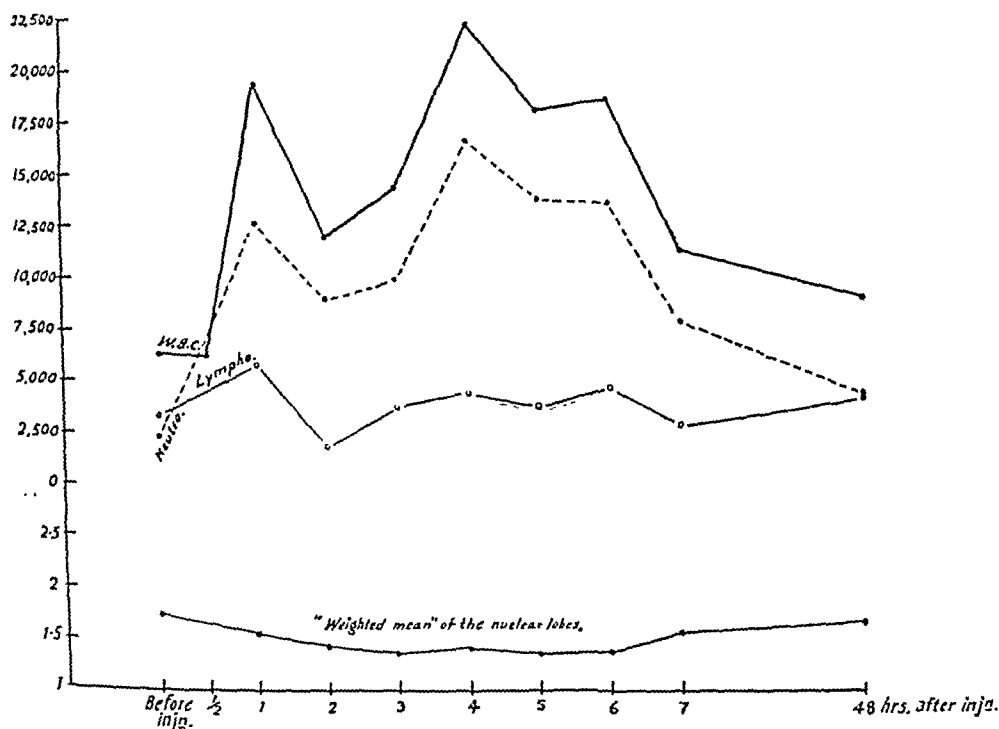
After single injection of 0.025 g. of acetyl-choline in a splenectomized rabbit.

Time.	W. B. C. total.	NEUTROPHILS.		'Weighted mean.'	LYMPHOCYTE.		Immature cell, per cent.
		Per cent.	Total.		Per cent.	Total.	
Before injection ..	9,800	43.0	4,214	1.74	52.5	5,145	<i>Nil.</i>
$\frac{1}{2}$ hour after injection ..	9,600	44.5	4,272	1.58	49.5	4,752	<i>Nil.</i>
1 " " " ..	25,440	55.0	13,970	1.48	33.0	8,382	<i>Nil.</i>
1 $\frac{1}{2}$ hours " " ..	12,600
2 " " " ..	9,300	60.0	5,580	1.55	34.0	3,192	<i>Nil.</i>
3 " " " ..	20,900	70.0	14,644	1.42	26.0	5,440	<i>Nil.</i>
4 " " " ..	28,200	71.0	20,022	1.42	23.0	6,456	<i>Nil.</i>
5 " " " ..	13,280	66.5	8,831	1.37	27.5	3,652	<i>Nil.</i>
6 " " " ..	12,600	72.0	9,072	1.45	24.0	3,024	<i>Nil.</i>

Comment.—It has been shown that parenteral administration of parasympathetic stimulants leads to a great increase in the peripheral white cell count. This leucocytosis can be divided into two distinct phases—one immediate, occurring in about one to one and a half hours, and the other later, occurring in about four to six hours after the injection. The immediate increase is probably due to re-distribution of the cells from the internal organs. In the case of acetylcholine this is brought about at least to a great extent by contraction of the spleen. This view is supported by the absence of any immediate great increase o

CHART 9.

After single injection of 0.000125 g. of doryl in normal rabbit.



white cell count in the splenectomized rabbit after acetylcholine. The comparatively small initial leucocytosis after carbaminoyl was probably due to a smaller contractile action of the drug on the spleen as compared with acetylcholine. The late leucocytosis after the injection in all cases was probably due to mobilization of pre-formed reserves from the marrow, as is evident from the occasional presence of immature cells and the lowering of the 'weighted mean' by increase in staff neutrophils in all the experimental animals.

TABLE IX.

After single injection of 0.000125 g. of doryl in a normal rabbit.

Time.	W. B. C. total.	NEUTROPHILS.		'Weighted mean.'	LYMPHOCYTE.		Immature cell, per cent.
		Per cent.	Total.		Per cent.	Total.	
Before injection ..	6,400	38.0	2,432	1.74	54.0	3,456	Nil.
$\frac{1}{2}$ hour after injection ..	6,320
1 " " " ..	19,400	65.0	12,610	1.56	30.0	5,820	Nil.
2 hours " " " ..	12,000	73.0	9,052	1.44	16.0	1,984	Nil.
3 " " " " ..	14,400	69.0	9,936	1.38	26.0	3,744	Nil.
4 " " " " ..	22,160	74.5	16,509	1.41	20.0	4,432	Nil.
5 " " " " ..	18,000	76.0	13,680	1.37	21.0	3,780	Nil.
6 " " " " ..	18,500	73.0	13,505	1.39	25.0	4,625	Nil.
7 " " " " ..	11,200	70.0	7,840	1.57	25.0	2,800	Mct 1
24 " " " " ..	9,000	47.5	4,275	1.68	45.0	4,050	..

Mct = Myelocyte.

Only a few experiments were done on the action of atropine and physostigmine on the blood.

7. ATROPINE.

Herick (1914) did not find any change in the leucocytes after ordinary doses of atropine, but Backman *et al.* (*loc. cit.*) found that atropine caused a great increase in leucocytes. I made two experiments on the action of atropine on the blood. Atropine sulphate 1/30 grain was injected subcutaneously in both animals. In one no appreciable change either in the total or differential white cell count was noted, while in the other slight leucocytosis was observed which reached the maximal point in about four hours after the injection. The leucocytosis was due to increase in both neutrophils and lymphocytes. No immature or young cell was found in either case (Chart 10, Table X).

CHART 10.

After single injection of 1/30 grain of atropine sulphate in normal rabbit.

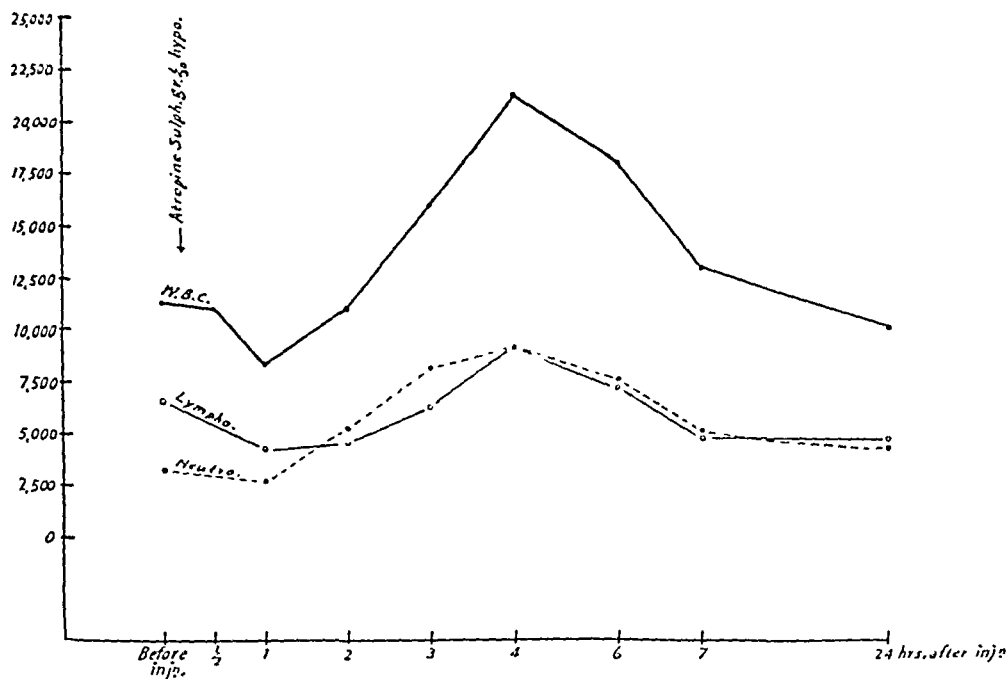


TABLE X.

After single injection of 1/30 grain of atropine sulphate in a normal rabbit.

Time.		W. B. C. total.	NEUTROPHILS.		'Weighted mean.'	LYMPHOCYTE.		Immature cell, per cent.
			Per cent.	Total.		Per cent.	Total.	
Before injection	..	11,300	30.0	3,390	..	58.0	6,554	Nil.
1/2 hour after injection	..	11,000
1 " " "	..	8,400	42.0	2,728	..	52.0	4,364	Nil.
2 hours " "	..	11,000	49.0	5,390	..	41.0	4,510	Nil.
3 " " "	..	16,000	48.0	7,680	..	40.0	6,400	Nil.
4 " " "	..	21,200	42.5	9,010	..	42.5	9,010	Nil.
6 " " "	..	18,000	42.0	7,560	..	40.0	7,200	Nil.
7 " " "	..	13,000	39.0	5,070	..	37.0	4,700	Nil.
24 " " "	..	10,000	41.5	4,399	..	44.0	4,664	Nil.

Comment.—The slight leucocytosis observed after atropine was probably due to re-distribution of cells from internal organs.

8. PHYSOSTIGMINE.

Two rabbits received injections of 1/6 grain and 1/20 grain of physostigmine subcutaneously—the injection was well tolerated. In both there was an increase in total white cells, which however did not sufficiently manifest itself till the fourth or fifth hour, and a return to the original level in about the sixth or seventh hour. The leucocytosis was entirely due to increase in the neutrophils. The increase in neutrophils was seen early, before there was any sign of increase in the total white cell count, and the increase in the neutrophils persisted even when the total count came to the normal level and returned to the original level in 24 hours. A fall in the lymphocytes was noticed from the first hour after the injection and the lymphocytes continued at a low level up to six to seven hours and came back to the pre-injection level after 24 hours. No immature cell was found at any time in either of the animals (Chart 11, Table XI).

CHART 11.

After single injection of 1/16 grain of physostigmine in normal rabbit.

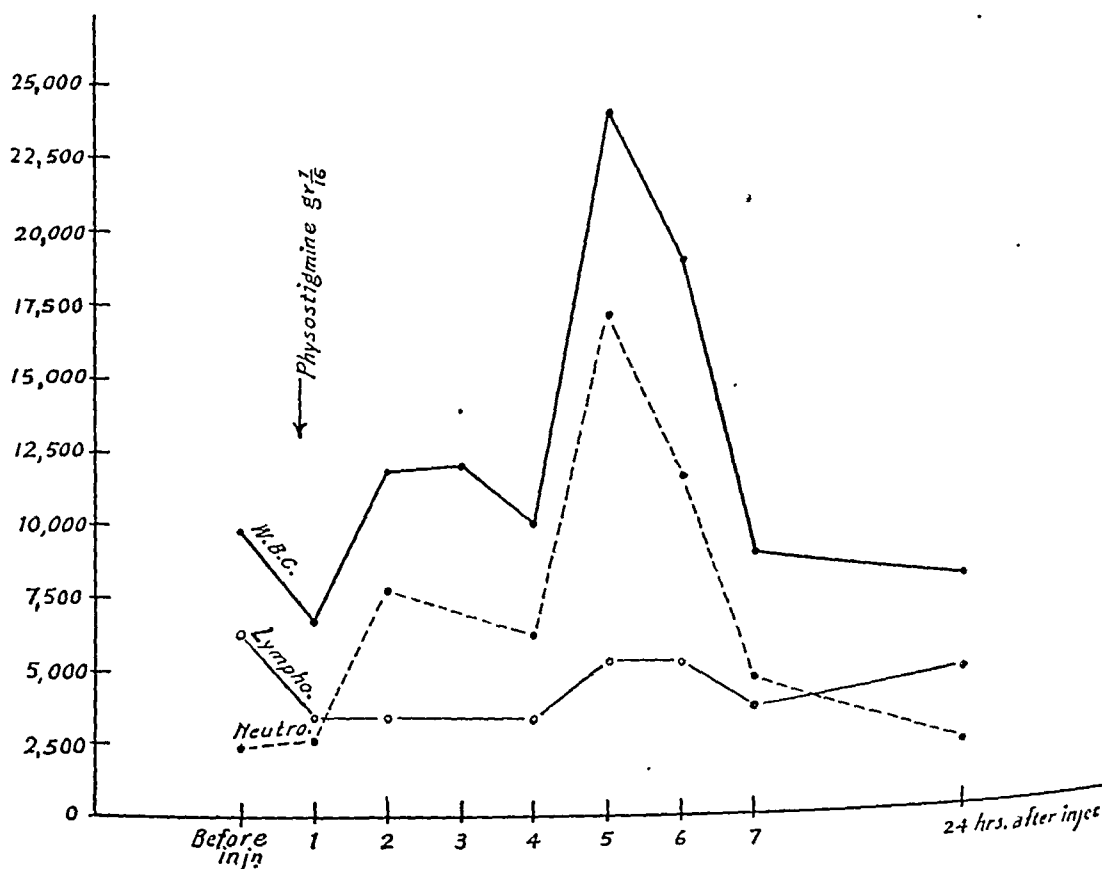


TABLE XI.

After single injection of 1/16 grain of physostigmine in a normal rabbit.

Time.	W. B. C. total.	NEUTROPHILS.		'Weighted mean.'	LYMPHOCYTE.		Immature cell, per cent.
		Per cent.	Total.		Per cent.	Total.	
Before injection ..	9,700	25.5	2,473	..	61.5	6,256	Nil.
1 hour after injection ..	6,600	38.0	2,508	..	53.0	3,498	Nil.
2 hours " " ..	11,800	66.0	7,788	..	29.0	3,422	Nil.
3 " " " ..	12,000
4 " " " ..	10,000	61.0	6,100	..	32.0	3,200	Nil.
5 " " " ..	21,000	71.0	17,040	..	22.0	5,280	Nil.
6 " " " ..	19,000	61.5	11,685	..	27.5	5,225	Nil.
7 " " " ..	9,000	53.0	4,770	..	40.0	3,600	Nil.
24 " " " ..	8,000	28.0	2,280	..	61.0	4,880	Nil.

In one animal, along with the leucocyte count, an estimation of hæmoglobin and red blood cell count was also done. There was a gradual rise of the red blood cells from 5 million to over 7 million in three hours and a return to the original level in five hours, though the hæmoglobin did not show any appreciable variation during the whole of the experiment.

Comment.—Nelson and Edmunds (1924) found that physostigmine produced an acute increase in red cell count, reaching a maximum in one hour and returning to the normal level in two to three hours, and they argued that it was probably an indirect result of the stimulation of smooth muscle which expressed blood from the stagnant areas. An increase in the red cell count was also obtained by me in one case but the maximum point was reached after three hours. Unfortunately I did not do the Arneth count in either case and it is not possible to say from the data in hand whether the increase was of bone-marrow origin or not.

DISCUSSION.

An increase in the polymorphonuclear leucocytes in the peripheral blood may be the result of (i) re-distribution of circulating leucocytes, (ii) mobilization of the granulocytic reserves of the bone-marrow, or (iii) increased maturation of granulocytes. In health more leucocytes are present in the blood of the viscera than at the periphery, and a variety of agents can induce temporarily a more uniform distribution of the white cells throughout the vascular system, probably as a result of capillary dilatation and contraction of capsular and trabecular smooth muscle. Re-distribution of the leucocytes may occur within a few minutes and subside in a

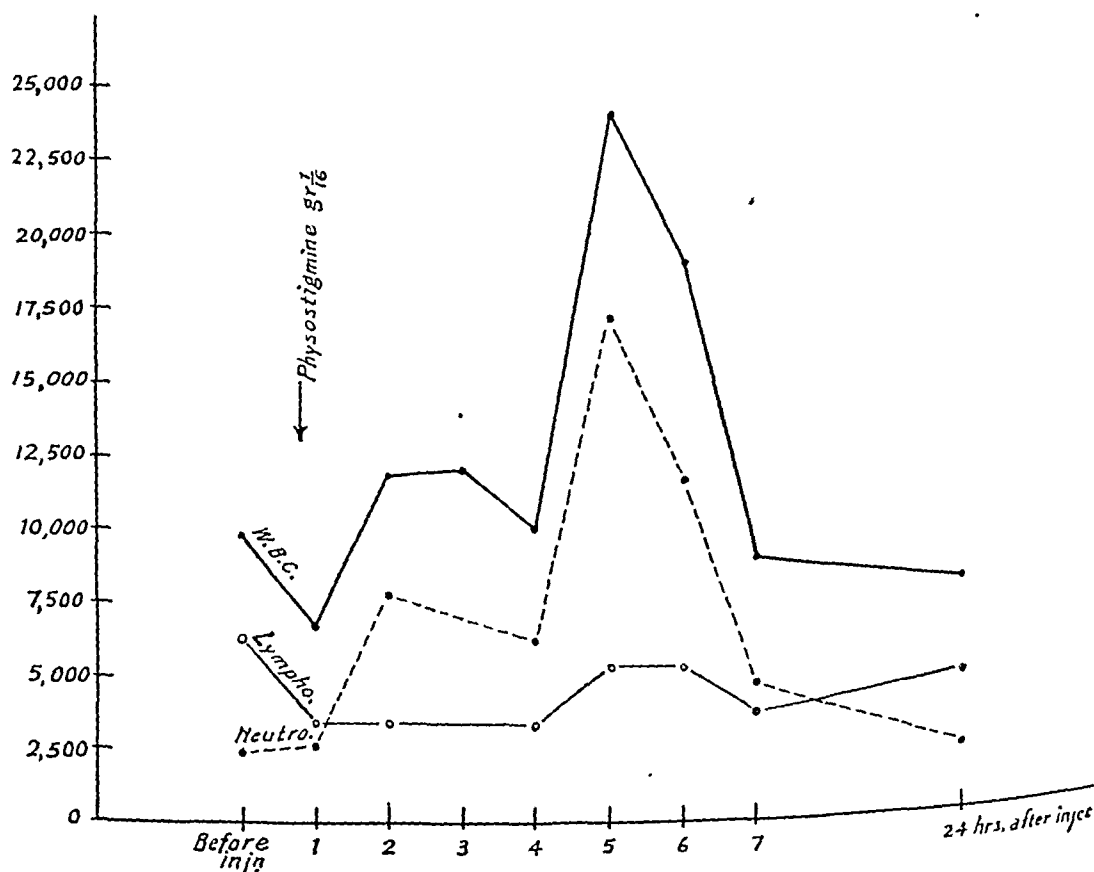
Comment.—The slight leucocytosis observed after atropine was probably due to re-distribution of cells from internal organs.

8. PHYSOSTIGMINE.

Two rabbits received injections of $1/6$ grain and $1/20$ grain of physostigmine subcutaneously—the injection was well tolerated. In both there was an increase in total white cells, which however did not sufficiently manifest itself till the fourth or fifth hour, and a return to the original level in about the sixth or seventh hour. The leucocytosis was entirely due to increase in the neutrophils. The increase in neutrophils was seen early, before there was any sign of increase in the total white cell count, and the increase in the neutrophils persisted even when the total count came to the normal level and returned to the original level in 24 hours. A fall in the lymphocytes was noticed from the first hour after the injection and the lymphocytes continued at a low level up to six to seven hours and came back to the pre-injection level after 24 hours. No immature cell was found at any time in either of the animals (Chart 11, Table XI).

CHART 11.

After single injection of $1/16$ grain of physostigmine in normal rabbit.



15 minute intervals, reported a characteristic rhythm and a definite rise in the afternoon counts which was independent of any digestive process. Shaw (1927) corroborated Sabin's findings in the main and found two high peaks in the course of 24 hours, one in the afternoon and the other at about midnight, these day and night tides occurring regardless of food. In the same way there is unanimity of opinion with regard to the leucocytosis produced by exercise. A short bout of severe exercise causes leucocytosis up to 100 per cent or more, while the increase is much more after long strenuous exercise, as has been shown in Marathon runners, in one of whom the leucocytes went up from 3,700 to 20,800 during a race (Garrey and Bragan, 1935). Even change in posture is supposed by many to produce leucocytosis and Cheng (1930) reports a difference in the leucocyte counts in rabbit dependent upon stomach-up or back-up positions. Psychic stimulation without any obvious muscular activity is also said to produce a certain amount of variation in the leucocyte count. Thus, the intense odour of food and aromatics and even the thought of food are sufficient to cause leucocytosis, and variation in the leucocyte count has been noted under different emotional conditions in rabbits, e.g., leucocytosis when teased (Cohnstein and Zuntz, 1888), relative monocytosis when excited (Menkin, 1928), and leucopenia when emotional (Nice and Katz, 1934). Leucocytosis observed before operation in uninfected cases has been attributed to fear or apprehension and even slight pain, such as the prick of a needle, may be sufficient to produce a leucocytosis, which has been regarded as being due to liberation of a histamine-like substance (Findlay, 1928). The leucocytoses noted under all the above conditions are physiological, being due to re-distribution of cells from centre to periphery without any change in the nuclear lobe count.

Knowing the wide range of leucocytic variation possible, even with the needle-prick and the slight manipulation necessary to take a sample of blood from an excitable animal like the rabbit, it is rather difficult for one to say with any degree of certainty whether the leucocytosis unaccompanied by any change in the nuclear lobe count, which has been noticed after many drugs, is due to the drug or not. But considering the great amount of leucocytosis noted after most of the drugs and taking in account that leucocytosis of this degree was never seen in the control animals, even after injections of an indifferent solution such as fresh normal saline, it is reasonable to conclude that the leucocytosis was probably due to the action of the drug. With the exception of Colchicine, the leucocytosis was more apparent than real and was attributable to re-distribution of the white cells in circulation and to more or less outpouring of pre-formed granulocytes from the marrow. In certain instances, as with adrenaline and parasympathetic stimulants, these two phases appeared to be represented by successive peaks in the white cell count. The leucocytosis was transient, and with pentnucleotide and liver extract it was impossible to produce a sustained rise in the white count or evidence of increased proliferation and maturation of the leucopoietic cells in the marrow, even though injections were frequently repeated. Colchicine undoubtedly stimulates the formation of new cells in the marrow, and induces immature cells of both the red and the white series to appear in the peripheral blood, but it is frankly toxic and its destructive powers outweigh its stimulant effects.

It may be considered disappointing and indeed surprising that I have found no evidence that pentnucleotide or liver can stimulate the new formation of leucocytes in health. Everyone accepts the fact that iron and liver will not raise the

red cell count beyond the normal level and *a priori* there seems no reason why the leucopoietic tissues should behave differently when they are supplied with an excess of hæmatinic principles. In the appropriate sections of this paper I have criticized previous work which had been thought to indicate that liver and pentnucleotide can stimulate the new formation and maturation of white cells in health. The immediate leucocytosis which follows the injection of these drugs is transient and more apparent than real. No enduring leucocytosis, no myelocytosis, and no outpouring of immature leucocytes follow their repeated administration, unless the experiments are complicated by major traumata, which are of themselves sufficient to account for any changes reported in the white cells. It may be that, like the liver principle in pernicious anæmia, pentnucleotide and liver act only in conditions of pathological leucopenia. My experiments allow me no right to speak on this matter though I may be forgiven for pointing out that evidence of their therapeutic value in agranulocytosis is not convincing (Witts, *loc. cit.*).

SUMMARY.

1. The ability of a number of drugs to produce leucocytosis, with special reference to their possible value in the treatment of agranulocytosis has been studied.

2. Leucocytosis may be due to (i) re-distribution of circulating leucocytes, (ii) mobilization of pre-formed reserves in the bone-marrow, and (iii) increased maturation of granulocytes. The third property only is likely to be of value in agranulocytosis.

3. Only transient leucocytosis, due mainly to re-distribution and partly to mobilization of leucocytes, was observed after single injections of pentnucleotide sodium nucleinate, liver extract, histamine, adrenalinic, and acetyl-choline and related substances.

4. No evidence was obtained that liver extract or pentnucleotide can accelerate the formation of white cells in health, even though injections are frequently repeated.

5. Colchicine stimulates the proliferation of cells in the marrow but the toxic effects of this drug outweigh its stimulant powers.

ACKNOWLEDGMENTS.

I wish to thank Professor L. J. Witts for constant advice and guidance in carrying out the experiments and in writing this paper; Miss A. Hartridge for technical assistance; the Medical Research Council, and Messrs. Smith, Kline and French Laboratories for unlimited supplies of pentnucleotide; and Messrs. Evans, Lescher and Webb for unlimited supplies of liver extract.

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A STUDY OF ANÆMIA AFFECTING LABOURERS ON ASSAM TEA-ESTATES.

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INTRODUCTION AND TECHNIQUE.

INTRODUCTION.

A FORM of anæmia occurs amongst the labourers on tea-estates in Assam which is the cause of much sickness and mortality. Its existence in this class of people has attracted attention because they are subject to more thorough medical supervision than is the case with any comparable population in India, and the economic results are more apparent than in the general population. Cases of a 'similar type are, however, seen amongst the general population.

As the result of some years of work on tea-estates the author has come to consider anæmia as the chief cause, with the possible exception of malaria, of sickness and death. Its importance has been fully realized by the planting community who appreciate the economic loss which it causes both to employer and employee. They have demonstrated their interest by the contribution of large sums of money to research on the subject, and the expenditure of larger sums on attempts at prophylaxis and treatment.

Despite this active interest, the incidence of the condition has remained almost unchanged for many years, and there was little advance in treatment until the work of Napier and Das Gupta (1937) proved the value of maximal iron therapy. There has still been little advance in prevention; the present work is an effort to discover any causative factors of the disease, a knowledge of which might be helpful in preventing it or reducing the damage done by it.

The pathology of the condition has been the subject of exhaustive study by Napier (1937) and Napier and Das Gupta (1935*a, b, c, d*; 1936; 1937) in several series of clinically anæmic and clinically non-anæmic persons. They describe a condition of anæmia prevalent amongst the entire population, whether apparently unhealthy or healthy, and the following description is derived from their work:—

The anæmia in the apparently normal population is characterized by a nearly normal erythrocyte count; a low hæmoglobin level, with a mean of 11·83 grammes hæmoglobin per 100 c.c. blood in men, and 10·03 grammes in women; and a correspondingly low colour index with a mean of 0·81 in both men and women. There is a marked reticulocytosis, with a mean of 2·17 per cent. Eosinophils and large mononuclear leucocytes are in excess of the normal, with means of 13·75 and 9·025 per cent of the total white cell count respectively. There is a significant difference between the sexes, anæmia being most marked amongst women, and an age difference, the anæmia being more severe amongst those under 30 years old, both men and women, than amongst those over that age.

In two series of clinically anæmic cases they found that the great majority were microcytic and hypochromic. A few orthochromic cases were seen. There were no distinctly macrocytic cases except in a series of pregnant women. Anisocytosis and polychromasia were common, and nucleated red cells were found in 13 per cent of cases. Reticulocytosis was more marked than in the clinically healthy group, the mean percentage of reticulocytes being 4·26 in one series and 3·92 in a second. Red cell fragility was decreased. The total leucocyte count, and the differential count, resembled those seen in the apparently healthy group. The direct serum van den Bergh test was negative in all cases; the indirect test was positive in 34 out of 87 cases, it was most frequently positive in the orthochromic cases and in those with enlargement of the spleen. The van den Bergh test on the urine was positive in a number of cases, and urobilinuria was frequently present. The results of gastric analysis were normal.

They further showed that hookworm infection was present in the entire population, but that heavy infestations were more common amongst the anæmic group than in apparently normal persons; there was no relationship, however, between the number of hookworm eggs found and the degree of anæmia in the anæmic group, and some highly anæmic persons showed low egg-counts.

The specific effect of ferrous salts in producing a rapid, but not complete, cure was demonstrated, even despite the presence of heavy hookworm infestation, which did not necessarily prevent the maintenance of a high hæmoglobin level for some months. The mean improvement in those with high original egg-counts was less than in those with low, though the difference was not considered significant. When the treated subjects were divided into two groups, those showing an increase of over 1 grammes hæmoglobin and those showing a smaller improvement, the first group contained significantly fewer cases with high egg-counts than did the second group. Also it was noted that on the estate on which anthelmintic treatment was least efficient, the hæmoglobin level was not maintained, whereas on other estates it continued to rise after the end of treatment.

The substitution of what was thought to be an ideal diet for the ordinary hospital diet for a period of a month, without specific treatment, did not produce any improvement in the blood picture of a series of cases. On considering the history of the various series treated, however, they considered that a good diet might have some effect on the maintenance of a high hæmoglobin level.

They concluded from the blood picture and the results of treatment that the anæmia was the result of an iron deficiency. The cause of the iron deficiency was not clear, it did not appear to be due to a deficiency in the diet, or to failure of absorption, and they considered that the chief cause was a blood loss due to hookworm infestation. They based their conclusions on the similarity of the blood picture to that seen in known hookworm disease, on observations on the relationship between the degree of infestation and anæmia, and of the effect of heavy infestations on the maintenance of a high hæmoglobin level.

Their exhaustive studies gave an exact account of the pathology and the treatment of the condition. The description of an ultimate cause, hookworm infection, was less certain, and did not explain all the various phenomena which they had observed. They suggested that possibly malaria and prolonged dietary defects might have some causative influence, but that there still remained some unknown factor limiting the final hæmoglobin level.

As the result of some clinical experience, and observation of the effects of treatment, the writer became impressed with the uncertainty of the position of hookworm infestation as to the major cause of the disease, and came to regard the evidence on which it is so considered as unconvincing. Hookworm infestation is common to the entire population; if it produces this condition which varies so greatly in degree, it is logical to assume that the variations must be due either to differences in the degree of infestation, or to the adjuvant effect of some other factor. In the first case an exhaustive study should have shown a closer relationship between the load of hookworm eggs in the stools and the degree of anæmia, and cure should have been more dependent on the efficacy of anthelmintic treatment. In the second case the adjuvant factor or factors would appear to have an importance equal to, or greater than, the hookworm infestation.

A study of other possible factors which might limit the hæmoglobin level was, therefore, considered desirable. Pathological investigation quite beyond the resources available to the author had not clearly shown them, though it had made their existence likely. The pathological approach was therefore not used; a study

was made of the epidemiology of the condition, and of its epidemiological background, in the belief that it would discover one or more other causative factors, and where some indications of such a cause were found, they were followed with the resources at the author's disposal.

An essential of any epidemiological description is an account of the background on which the condition occurs, an account of the general health of the population, its vital statistics, and the diseases which affect it and might be related to it. This is more particularly desirable in describing a condition occurring in Assam, as the Census Commissioner considers the official vital statistics to be grossly inaccurate, and there are few detailed accounts of the incidence of disease amongst the population.

The study has been arranged in five parts. PART I, introduction and technique, PART II is an account of the vital statistics and the general incidence of disease, PART III describes the incidence of anæmia, its distribution amongst the population, and the mortality and morbidity due to it, PART IV gives an account of the relationship between anæmia and diet, with special reference to the different varieties of rice consumed, and PART V discusses some evidence accumulated to show the relation between anæmia and malaria. Unfortunately this portion of the work was abruptly terminated before completion by the departure of the author from Assam.

Although the work has been ended suddenly the material for the first four parts had been accumulated, and had demonstrated hitherto unknown elements in the ætiology of the disease, and the study composing the fifth part had disclosed an apparent connection between the two diseases, which was inconclusive but yet justified serious consideration. The whole is, therefore, published despite its unfinished form.

TECHNIQUE.

The homogeneous nature of the universal anæmia has been demonstrated by Napier and Das Gupta. In the present investigation, therefore, anæmia has been considered as a condition in which the hæmoglobin was below the normal standard, and no effort at further diagnosis or pathological examination made. This is justified by the fact that a part of the work of these authors was done in the same population as that here dealt with.

Hæmoglobin was estimated by two methods: the Tallqvist scale and the Hellige apparatus. No apology is made for the use of the Tallqvist as the results here given include about 10,000 estimations, which could not have been made by any other method, but some explanation of the reliance placed on the results obtained by it is indicated. Much use has been made of the calibration and evaluation of the two scales by Napier and Das Gupta (1935a).

In the Tallqvist scale finger blood is absorbed into a piece of especially prepared blotting-paper, and the resultant colour compared with a standard on opaque paper; estimations are made in percentages, and always in multiples of five. This is clearly an inaccurate method. Napier and Das Gupta compared Tallqvist and Hellige estimations, and found that the former had a standard error, for individual readings, of 11·8 per cent. In the higher parts of the scale, above

50 per cent, there was also considerable divergence of the two scales and the error was large. The error was smaller below 50 per cent and the two scales more nearly approximated each other.

It is obvious that no reliance can be placed on individual readings except in the most general way. However, when large numbers of readings are taken it gives three valuable indications; if an individual reading is 50 per cent or less, the assumption that the hæmoglobin really falls into this class is a fairly sound one, and the numbers shown to have these low readings may be taken as reasonably accurate; the mean of a large number of readings, not less than 100, may be relied upon and compared with the mean of other similar series; similarly, a frequency distribution based on a very large number of cases, not less than 1,000, may be used in statistical analysis. In this paper all three of these types of results are tabulated, but only the first, which is probably the most reliable, was used in statistical analysis.

Individual readings by the Hellige have a standard of error of 1.4 per cent; 100 per cent on this scale corresponds to 13.67 grammes hæmoglobin per 100 c.c. blood. All types of results, including individual readings, are statistically usable. Finger blood was used in all investigations and the technique of Napier and Das Gupta closely followed.

No response has been made to the suggestion made by these authors that hæmoglobin readings should be given in terms of grammes hæmoglobin per 100 c.c. of blood and not in percentages. The conversion of readings taken in one type of unit (percentage) into another type of unit (grammes Hb) and the subsequent preparation of frequency tables in terms of the second unit leads to false groupings which may lead to serious confusion. A frequency table based on 0.5 gramme hæmoglobin differences will contain three percentage readings in some groups, four in others. For instance, the group Hb 8.00 g. to 8.49 g. contains the Hellige percentage readings 59, 60, 61, and 62; that of 8.50 g. to 8.99 g. the readings 63, 64, and 65. Inaccuracy can only be eliminated by making the groups unnecessarily large, or by making the groups correspond to the units in which the measurement was originally made; thus a grouping of 8.20 g. (60 per cent) to 8.82 g. (64.5 per cent) would not lead to error.

This is not a hypothetical point. A significant grouping, in grammes of hæmoglobin, in this study disappeared when the readings were converted back into percentage units. A compromise has been made by giving readings in terms of Hellige percentage units, and stating the equivalence at the foot of each table.

The only other technique extensively used has been that of the census. It has been a part of the routine medical administration of these estates to hold an annual census and medical examination. The census held in the winter of 1936-1937 was considerably elaborated. In its elaborated form it included an inquiry from each person of name, age, sex, caste, or race, duration of residence in Assam, the variety of rice normally consumed, and other details concerning housing accommodation and sleeping habits, and an estimation of the hæmoglobin by the Tallqvist scale. The books were prepared in such a way that families could be distinguished.

The normal census covered a population of about 15,000 people. For a number of reasons the elaborate census could not be extended to all estates and covered 9,042 persons.

Details were taken by Assistant Medical Officers, who themselves estimated the age. A study of the results showed a marked preference for certain age period and avoidance of others, by different estimators, so that in classification the simple grouping of under 1 year, 1 to $4\frac{1}{2}$, 5 to $9\frac{1}{2}$, 10 to $14\frac{1}{2}$, 15 to 44, 45 and over, was all that could be used. The information concerning duration of residence in Assam was probably fairly accurate in most cases, and was grouped as under 3 years, 3 years and under 6, 6 years and under 10, 10 years and under 20, over 20 years, and born in Assam. The classification of the first two groups in periods of three years was used to correspond with the normal period of short-term recruitment.

During later medical examinations the hæmoglobin of a random selection of about every twentieth person was checked by the author ; in addition every person recorded as having a low hæmoglobin, or suspected to have one on clinical examination, was also re-examined by the author.

The variety of rice consumed is discussed in a later part of this paper. The question was asked of each person, or his guardian, and the answer recorded. There was no reason why any person should wish to name, or deny, any variety.

It is obvious that this detailed inquiry would have been impossible without the willing and interested help of several assistants, who ungrudgingly gave a great deal of their time to the work, and to whom the sincere thanks of the author are due.

Part II.

THE POPULATION STUDIED. ITS VITAL STATISTICS, AND THE CHIEF CAUSES OF SICKNESS.

THE POPULATION.

The population studied was that of a group of tea-estates in the Sibsagar district of Assam, of which the latitude is 26° North, and longitude 94° East. The district forms part of the level alluvial valley of the Brahmaputra. The elevation is about 500 feet above sea-level, but as the nearest sea-coast is over 300 miles distant in a straight line, and over 700 miles along the course taken by the Brahmaputra, the effect of elevation on land drainage is not noticeable. The climate is continental and sub-tropical, with a marked cold season and a very hot summer. The atmospheric saturation with water vapour is high at all times of year, and the chief rainfall occurs during the months between April and October. The principal meteorological averages for each month are shown in Table I :—

TABLE I.

The average temperature, humidity, and rainfall for Sibsagar for each month of the year.

Month.	TEMPERATURE.		Relative humidity mean 8 a.m.	Total rainfall in inches.
	Mean maximum, °F.	Mean minimum, °F.		
January	70.0	49.7	98	1.29
February	72.6	53.3	96	2.01
March	78.6	59.8	91	4.78
April	81.2	65.9	90	10.11
May	85.4	71.5	90	11.89
June	88.7	76.1	91	14.21
July	89.2	77.7	92	17.01
August	88.6	77.6	93	16.27
September .. .	87.6	76.2	93	11.70
October	84.1	70.6	95	5.10
November .. .	77.7	59.6	96	1.10
December .. .	71.1	50.6	96	0.52

believes to show the effect of the steady improvement in general hygiene on men at an age when the epidemic causes of death do not much affect them. The mean death rate for women aged 15 to 45 is 9·3 per 1,000 higher than that for men of the same age, and the author believes that almost the entire difference is due to the direct or indirect effect of anæmia, which affects this age and sex group more markedly than any other.

Table IV lists the causes of 1,231 deaths on ten estates, on which all deaths were certified by a registered medical practitioner. They have been listed in a simple classification which makes clear the more important causes. Respiratory diseases and bowel diseases are the two most important causes of death. Malaria and anæmia are almost equal. The number of deaths due to childbirth, and of infant deaths due to prematurity and to undetermined infantile conditions are noteworthy. These last three causes of death are believed to be very largely due to anæmia.

A detailed explanation of the reasons on which this belief is founded is given in Part III of this paper.

TABLE III.

The death rate by age and sex groups of a population averaging 11,069 persons.

Year.	AGE GROUP.					TOTAL.
	0-1.	1-4½.	5-14½.	15-44.	45 and over.	
Males.						
1933 ..	247	46·2	9·38	14·84	46·6	29·9
1934 ..	261	81·5	9·78	13·4	40·8	33·4
1935 ..	142	56·7	13·8	13·0	46·4	27·8
1936 ..	259	52·5	12·9	12·5	52·1	32·5
AVERAGE ..	225	59·2	11·6	13·4	48·1	30·8
Females.						
1933 ..	275	36·3	13·8	23·0	21·4	31·1
1934 ..	185	58·6	19·8	18·9	45·1	32·8
1935 ..	137	33·7	11·8	24·8	43·8	28·7
1936 ..	208	53·1	12·3	23·4	34·9	33·1
AVERAGE ..	198	45·3	14·3	22·7	39·0	31·38

TABLE IV.

A list showing the annual number of deaths, classified according to the cause of death, in a population averaging 9,912 persons.

Cause of death.	1933.	1934.	1935.	1936.	Total.	Rate per 1,000 per annum.
MALARIA	48	20	22	30	120	3.03
RESPIRATORY DISEASES						
Pneumonia	31	18	32	28	109	2.75
Pulmonary tuberculosis	8	6	16	15	45	1.13
Other diseases	15	48	31	32	126	3.18
BOWEL DISEASES						
Choleraic diarrhoea	5	6	12	11	34	0.86
Dysentery	15	24	32	26	97	2.45
Other diseases	13	15	16	13	57	1.44
ANEMIA	33	31	27	27	118	2.98
CHILDBIRTH						
Sepsis	5	1	2	7	15	0.38
Other causes	5	6	9	10	30	0.76
ASCARIASIS	1	16	10	8	35	0.88
NEPHRITIS	4	10	9	12	35	0.88
SURGICAL DISEASES	2	2	1	7	12	0.30
SEPTIC CONDITIONS	10	7	4	2	23	0.58
ZYMOtic and INFECTIOUS DISEASES.	6	17	17	5	45	1.13
PREMATURITY OF BIRTH	15	28	11	19	73	1.84
UNDETERMINED INFANTILE CONDITIONS (marasmus and asthenia).	35	28	19	26	108	2.72
CAUSE UNKNOWN	3	8	6	10	27	0.68
OTHER CAUSES	28	25	31	38	122	3.08
TOTAL	282	316	307	326	1,231	31.05

THE CHIEF CAUSES OF SICKNESS.

In the following account an effort has been made to give figures for the incidence of disease as far as possible. They are based on the population for which a standard system of record keeping was maintained, which included 10,284 persons in 1933, 12,303 in 1934, 15,185 in 1935, and 14,810 in 1936.

In the opinion of the author the chief entity responsible for sickness and death is anæmia. Figures showing the incidence of the disease, and the number of cases treated, are available, but though imposing are almost meaningless. Most anæmia cases are discovered and brought under treatment as a result of a systematic search for cases, and not of their own volition. The number of cases treated is, therefore, more likely to reflect the efficiency of the search than the incidence of the disease. To demonstrate the true situation reliance must be placed on hæmoglobin surveys of the whole population, and on an estimate of the importance of the condition as a cause of death, rather than on the number of cases treated. This subject will be developed in Part III of this paper.

Malaria ranks almost equal to anæmia as a cause of sickness. An account of the incidence of malaria on these estates has been given by Macdonald and Chowdhury (1931); transmission is exclusively by *Anopheles minimus*, and takes place normally from the middle of March to the middle of November, with possibly slight transmission throughout the year (Rice and Mohan, 1936). All three species of parasite are found, *Plasmodium falciparum*, constituting 72 per cent of infections, *Plasmodium vivax* 20 per cent, and *Plasmodium malariae* 5 per cent with 3 per cent of mixed infections. Malaria control measures were started on some estates in 1933, and the incidence of cases per 1,000 population fell from an unknown high figure to 499, 373, 315, and 340 respectively in the years 1933 to 1936. These are not reliable figures, owing to variations in the method of diagnosis and registration. A true picture is shown by the spleen rate, the variations in which are shown in Table V:—

TABLE V.

Variations in the spleen rate, 1930 to 1936.

Year.	Highest.	Lowest.	General rate.
1930 ..	93	20	51·0
1933 ..	86	18	47·5
1934 ..	73	13	33·9
1935 ..	63	11	26·2
1936 ..	64	7·5	28·2

Respiratory diseases are the next most common cause of serious illness. Minor coughs and colds are, as in most countries, the commonest cause of absence from work. In addition to these, pneumonia, influenza, and bronchitis are common, and pleurisy and empyema occur. The general incidence of pneumonia throughout the period was 9.4 cases per 1,000 population per annum, the figures for successive years being 9.6, 7.6, 9.4, and 10.6. Other respiratory diseases receiving indoor treatment numbered 37.6 per 1,000 per annum for the whole period, and 41.0, 38.4, 39.8, and 32.0 in successive years. The mortality from respiratory diseases generally was 7.06 per 1,000 per annum.

Amoebic dysentery is endemic in the population, 9.0 cases came to light per 1,000 population per annum, the rate in each of the four years being 9.0, 9.5, 10.5, and 6.8.

Bacillary dysentery is of frequent occurrence in epidemic form, mainly during the months of April, May, and June. Though sporadic cases occur throughout the year, epidemics are rare except during these months. The average annual incidence was 15.1 per 1,000 population per annum, and in successive years 15.0, 13.9, 18.3, and 12.9.

There are two distinguishable types of outbreak, water-borne and those which are transferred by contagion, probably through the mediation of flies. On two estates on which transmission was believed to be water-borne, new water supplies safe from pollution were installed, with a marked immediate result, the sporadic cases of dysentery almost entirely ceasing. The main epidemics, however, appear not to be water-borne, transmission is probably through the agency of flies or direct contamination of food; they occur largely during those months when flies are most numerous, and it has been possible to trace the passage of an epidemic from person to person in the manner which one would expect of a highly contagious disease. Such epidemics may be localized in one corner of an estate, the whole of which draws its water from one source.

Ankylostomiasis is almost universal. The work of Napier and Das Gupta, which has already been referred to, was undertaken in this district, and partly on the population of the estates which are the subject of this inquiry. In an effort to control anaemia all members of the population have received at least one anthelmintic treatment annually for several years.

Ascariasis is universal amongst children, in whom it is at times a cause of death. Ill effects are not commonly seen in adults.

Cholera did not occur in the population in the years under review, but owing to the occurrence of cases in the district some thousands of persons were inoculated with vaccine during 1937. The cases of 'choleraic diarrhoea' which are relatively common as a cause of death appeared to be a severe form of bacillary dysentery, with which disease they are commonly associated.

The only zymotic disease of sufficient prevalence to be important was measles. A severe epidemic visited the district in 1933 and 1934, and caused many deaths, particularly in the age group 5 to 15 years.

All members of the population are vaccinated either at birth, or on their first arrival in Assam; in addition re-vaccinations are common. No smallpox cases occurred amongst the population of the estates during the years under review.

The district was not greatly affected by the last great epidemic of kala-azar, and is not known ever to have suffered severely from this disease. A constant watch was maintained for the disease, and many hundreds of aldehyde reactions performed; during the years 1935 and 1936 almost all cases of intractable anæmia were examined in this way. An average of only six positive cases were found annually, and these not amongst persons primarily complaining of anæmia.

Phrynoderma, xerophthalmia, night blindness, and other evidences of vitamin-A deficiency are of very frequent occurrence. It is clear that shortage of this substance is a frequent cause of ill health, but no relationship to anæmia was suspected. No evidence of deficiency of the vitamin-B complex was seen, a fact which is probably partly explained by the universal use of home-brewed rice beer, which is consumed regularly by persons of all ages.

Clinical evidences of a deficiency of vitamin C are extremely rare. The author has only seen two patients whom he believed to be suffering from scurvy, the two children of a destitute beggar who were brought on to an estate as an act of mercy.

Opium is very commonly consumed, most of the addicts being adult men, though some women acquire the habit. Accurate figures of the number of consumers are difficult to collect, but careful inquiry on a number of estates suggested that the habit was more prevalent on some than on others, and that the proportion of addicts varied from 0·5 to 5 per cent of the adult male population. Illicit opium is more commonly consumed than the legally acquired drug, and the amount consumed in each month rarely exceeds two tolas (360 grains), because the purchasing power of the coolie is limited and the normal cost of illicit opium is Rs. 4 per tola. No relationship between the consumption of opium and the incidence of the common anæmia could be traced; examination of a large number of addicts and their families suggested that the principal deleterious effect was not directly due to the opium, but to the deprivation of the necessities of life following the utilization of a large part of the total earnings in the purchase of the drug.

Indian hemp is commonly smoked, both legally purchased and illicit hemp being used. Symptoms of chronic respiratory disease and some mental aberration are the chief permanent ill effects.

Spirits are not regularly consumed except by a small minority. Almost all the population, however, consume regularly a form of home-brewed rice beer fermented for a couple of days.

Part III.

THE INCIDENCE OF ANÆMIA, AND THE MORBIDITY AND MORTALITY RESULTING FROM IT.

THE INCIDENCE OF ANÆMIA.

The method by which the census was taken and the technique and accuracy of the Tallqvist method of hæmoglobin estimation have been described. The hæmoglobin findings taken at the census are set out separately for men, women and children in Tables VI and VII. In Table VI actual figures are given, and in Table VII the same figures are reduced to percentages of the total in each class.

The frequency distributions show a very marked dispersion of readings about the mean and mode, entirely different from the closely agglomerated findings resulting from the accurate examination of a healthy population. A marked dispersion in the higher ranges would be expected if a healthy population were examined by the Tallqvist method owing to the inaccuracy of the technique, and can only be accepted as correct on account of its confirmation by estimations by the Hellige instrument which are described in Part IV of this paper. The dispersion in the lower ranges of the scale cannot be explained except by the presence of a common anæmia; a calculation based on the comparison of the Hellige and Tallqvist scale made by Napier and Das Gupta (1935*a*) shows that it is probable that 81 per cent of the persons described as having a hæmoglobin of 50 per cent or less did in fact fall into that category, and that the true number in that class was not less than 94 per cent of the stated number.

It will be noted that anæmia is prevalent amongst men, women, and children, but that the incidence of the severe forms is greatest in women, and least in men. If 'severe anæmia' is defined as corresponding to a Tallqvist hæmoglobin value of 50 per cent or less, then 10·42 per cent of women, 5·33 per cent of children, and 2·77 per cent of men were severely anæmic.

The higher incidence of anæmia in children than in men was only very slightly due to a differential sex incidence in children, in whom the ratio of severely anæmic females to males was 1·16 to 1. The ratio of the two sexes in the general population of children was 1·01 to 1. The slightly higher incidence amongst female children was entirely amongst the older ones, the two sexes being equal under the age of 12 years.

The modal hæmoglobin of each group is the same, 70 per cent, and is exceeded by 19·33 per cent of women, 19·37 per cent of children and 40·82 per cent of men. The Tallqvist scale is extremely uncertain in the higher ranges, but on account of the large numbers examined the numbers exceeding the modal 70 per cent may be taken as representative of the truth. It roughly shows the proportion of the population in whom the hæmoglobin value is satisfactory.

TABLE VI.

Showing the frequency distribution of hæmoglobin values in a population of 9,042 men, women, and children, in actual figures.

Hb (Actual figure.)	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	Total.	Mean.	Standard deviation.
Men	1	1	4	5	13	25	32	73	233	380	904	836	283	35	1	2,826	70.09	7.28
Women	2	3	18	27	41	77	110	150	413	486	821	454	58	3	..	2,663	65.03	9.69
Children	1	2	6	15	21	45	99	167	608	748	1,146	613	76	6	..	3,553	66.31	8.14

TABLE VII.

Showing the frequency distribution of hæmoglobin values in men, women, and children, in percentages of the total number in each group.

Hb (Percentage of total number.)	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90
Men	0.04	0.04	0.14	0.18	0.46	0.88	1.13	2.58	8.25	13.45	32.00	29.52	10.02	1.24	0.04
Women	0.07	0.11	0.68	1.01	1.53	2.89	4.13	5.63	15.51	18.23	30.83	17.04	2.18	0.11	..
Children	0.03	0.06	0.17	0.42	0.59	1.27	2.79	4.70	17.11	21.05	32.25	17.26	2.14	0.17	..

Table VIII was prepared in order to illustrate the relative incidence of anæmia in settled and newly arrived coolies. In order to avoid errors due to the different age and sex constitutions of the populations, adult women only have been counted here.

TABLE VIII.

The incidence of anæmia in new and settled women.

Estate.	SETTLED WOMEN.			UNSETTLED NEW WOMEN.		
	Number.	Mean Hb.	Percentage severely anæmic.	Number.	Mean Hb.	Percentage severely anæmic.
1	195	63·1	25	116	68·7	12
2	101	58·1	21	40	59·1	23
3	187	62·3	14	42	66·7	19
4	255	61·8	3	2
5	195	65·4	9	45	63·1	13
6	47	61·3	19	19	58·7	16
7	88	60·6	10	7
8	154	64·3	14	69	60·2	22
9	655	68·8	4	18	67·6	..
10	124	69·2	9
11	198	61·7	16	17	63·2	6

It is a common belief, shared by the author until he analysed these figures, that anæmia is more common amongst new coolies than amongst settled ones, and in fact it is often regarded as a disease primarily of new coolies. The present analysis affords no support for this view. If the percentage of severely anæmic persons in the two groups is compared for each estate it will be found that only in one (No. 1) is there a marked difference, and in that the percentage is significantly higher amongst settled women than new arrivals. In no other estate is there a difference which might not be expected as a result of pure chance. The reason for the significant difference in estate No. 1 is not known.

A second common belief is that all estates are essentially equally affected by the disease. The table gives very clear evidence to the contrary. There are great variations in both the mean hæmoglobin value and the percentage of severely anæmic cases. Statistical analysis shows that variations so marked as these could only be expected to occur by chance once in many millions of trials. It is beyond imagination that the differences shown are anything but real, and ascribable to some definite cause other than chance.

There is no true evidence of any seasonal evidence of anæmia. There is a very marked fictitious seasonal incidence with a peak in December and January. This is due to the fact that it is convenient to conduct an examination of the whole population during those months, and not due to any true seasonal variation in the disease. Clinical experience does not suggest any marked variation throughout the year.

MORBIDITY AND MORTALITY DUE TO ANÆMIA.

For most diseases to which reference has been made a figure has been quoted giving the annual number of cases treated. This is possible in the case of diseases with a rapid onset leading to early disability, which inevitably causes the patient to report for treatment. The case is quite different in anæmia; the onset is usually insidious, and at times extremely slow; at first the degree of disability is slight, the sufferer accommodates his life to his lessened powers, and will usually continue to work until he is on the verge of collapse, when his hæmoglobin may be found to be of the order of 25 per cent. On the estates on which this work was done, and on most estates, it has been the practice for some years to try to identify cases before they reach this stage, to bring them under treatment in hospital, and to keep them under treatment until a satisfactory recovery has been made. Under these circumstances statistics of morbidity represent the energy with which search has been made for cases, the success in inducing them to undergo treatment, and the interpretation of the words 'satisfactory recovery' more than they do the true incidence of the disease.

A calculation based on the known rate of improvement under ideal treatment shows that the 928 persons found at the census to have a hæmoglobin level below 60 per cent would require an average of 33 days' treatment each to reach that level, which is considered the lowest at which discharge from hospital should be allowed. This is equivalent to over 30,000 days' treatment distributed over the total population of nine thousand people. This figure represents an enormous loss of working capacity to the labourer, and financial loss to the employer. It is not, however, an exaggeration. It represents the ideal condition which would be achieved if it were possible to induce every one of these persons to accept in-patient treatment at once, and to accommodate them. In practice there is considerable additional loss of efficiency in those who refuse treatment, or delay acceptance.

Treatment on this scale has been practised annually for several years; in addition cases are sought throughout the year, and some cases report for treatment before they are discovered. The figures here given are thus considerably better than would be the case in an untreated population, and the loss of work to be contemplated is an annual one.

The mortality due to anæmia is also difficult to assess directly. Anæmia may cause death in a number of ways. Heart failure is the usual mode of death, and a number die suddenly from simple anæmia without any complicating disease, either because they have escaped observation and treatment, or despite treatment. These account for 2·98 deaths per 1,000 population per annum. Under any condition of abnormal stress a person who is anæmic may collapse and die. Sudden collapse is most common either during or immediately after childbirth, when the death is usually attributed to childbirth rather than to the true original cause, anæmia. This

is the most common cause of death in childbirth, other complications being relatively rare, as the normal small size of the child at birth makes obstructed labour uncommon.

There is no available method for calculating even roughly the number of people whose deaths, due to other diseases, would not have occurred if they had not been complicated by anæmia.

A common result of anæmia is premature childbirth. It is not usual to secure from a mother any accurate history of the duration of pregnancy, so the degree of prematurity is difficult to gauge, but amongst children of anæmic women, birth-weights of two and three pounds are very common, and less common amongst the children of healthy women. Living children of as low a weight as one and a quarter pounds are not unknown. Unexplained deaths amongst these children shortly after birth are usually attributed to prematurity, whilst if death is delayed for more than a few days, it is usually described as due to marasmus or asthenia. In the absence of any accurate data on the subject, the author would attribute three-quarters of the deaths ascribed to prematurity, and half of those ascribed to marasmus and asthenia, to anæmia of the mother. If this is correct, these two conditions would account for 1·48 and 1·36 deaths per 1,000 population per annum.

The death rate amongst adults may be estimated in another way, though in this again there is a considerable element of clinical belief to introduce uncertainty. The very marked excess of deaths amongst women as compared with men in the age group 15 to 45 may be seen in Table III. Without any doubt anæmia is a very large cause of this difference, and the author believes that it accounts for it practically entirely. This difference amounts to an annual death rate of 2·79 per 1,000 of the total population.

From this type of indirect evidence, a moderate estimate may be made that in a population of 1,000 people, the annual loss of working time as a result of anæmia exceeds 3,000 days, and the annual loss of life is of the order of six persons, three or four adults, and two or three infants.

Part IV.

DIET AND ANÆMIA.

THE DIET NORMALLY CONSUMED.

The staple food of all these labourers is rice, with the constant addition of 'dhal' (*Cajanus indicus*, a pulse resembling the lentil), and small quantities of mustard seed oil. Most people also secure some green vegetable and some form of animal protein on one or more days of the week. Tables IX and X illustrate the types of diets consumed. For the preparation of these analyses an Assistant Medical Officer collected details of the total diet of each family for six days, and they were then divided by six and by the co-efficients of the Technical Commission of the League of Nations Health Committee (1936) to represent the average quantity consumed per man per day. The errors of this form of calculation are fully appreciated, but it serves as a convenient way of describing an average diet, and no exact reliance is placed on the figures produced.

It is not in practice possible to discover the diet consumed by the average family on the seventh day of the week, when almost all people travel to the local market to make their weekly purchases and to enjoy themselves. It would include a quantity of rice beer, and some variety of sweetmeats and delicacies. A smaller quantity of rice beer is normally consumed throughout the week, but no account has been taken of its nutritive properties in the analyses given.

TABLE IX.

Diet consumed per man per day. Quantities are given in grammes.

Group.	Rice.	Dhal.	Oil.	Onion.	Green vegetable.	Potato.	Meat.	Fish.
Family 1 ..	800	44	4.6	0.3	41	20	..	3.2
Family 2 ..	795	66	10.0	7.6	46	..	66	..
Family 3 ..	765	27	8.6	12.5	64	33	69	..

The family consuming diet (1) consisted of a man, wife, and five dependent children. Diet (2) was that of a man, wife and two children, one of whom was at the breast. Diet (3) was that of a family of three adults and one dependent child.

TABLE X.

Showing the analysis of the three diets given in Table IX. Analyses according to Aykroyd (1937).

Constituent.				QUANTITY CONSUMED PER MAN PER DAY.		
				Diet (1).	Diet (2).	Diet (3).
Carbohydrate	549	659	627
Protein (vegetable)	68.0	74.0	73.3
Protein (animal)	0.5	22.2	15.7
Fat (vegetable)	(grammes)	8.4	16.4	14.7
Mineral matter	8.1	10.4	9.3
Calcium	0.22	0.20	0.15
Phosphorus	2.0	2.5	2.4
Iron (milligrams)	24.3	30.4	26.6
Calories	2,514	3,066	2,914
Carotene (International vitamin-A units)	1,162	1,442	1,040
Vitamin A	"	"	..	1.0	31	41
Vitamin B ₁ (International units)	789	979	900
Vitamin B ₂	present.	present.	present.
Vitamin C (milligrams)	24	28	30

The diets are open to criticism on a number of grounds; apart from the results of analysis they have the great disadvantage that there is no variation in the foods which supply the greater part of the dietary requirements, and that there is but small variation in the minor items. The analyses, when compared with the standards suggested by Burnet and Aykroyd (1935) and the Technical Commission of the League of Nations' Health Committee (1936, 1938) show a number of peculiarities which are too great to be explained by the method of computation.

The calorie value of all the diets is satisfactory, and in each case is derived mainly from carbohydrates. The total protein intake does not fall far short of the usual standards, though the quantity of animal protein is less than desirable in diets (2) and (3), and negligible in diet (1). The amount of fat eaten is very much below the normally accepted standards, but as there does not appear in the literature any clear account of ill effects resulting from a low fat intake this is possibly of little importance, especially as almost the only fat consumed is, in any case, a vegetable fat with no vitamin content.

The mineral content is chiefly peculiar for the very low calcium intake, which in each diet is less than half the minimum desirable intake, and less than one-third of the ideal requirement. On the other hand, phosphorus is present in relatively large quantities, greater than the necessary minimum, and the P:Ca ratio is exceptionally high; it should be remembered, however, that the phosphorus content of cereals is said to be largely in a non-assimilable form.

Iron is present in each of the three diets in apparently sufficient quantities, as it was in each of a number of other coolie diets which were analysed. Burnet and Aykroyd (*loc. cit.*) quote a tentative standard of 15 milligrams a day, and Aykroyd (*loc. cit.*), in discussing Indian diets, suggests that 20 milligrams should be adequate, while the League of Nations Committee's standard is 10 milligrams. It is

interesting to compare these figures with those found by Davidson *et al.* (1933), who investigated the diets taken by a number of poor Aberdeen women, and correlated the findings with the incidence of anæmia. The average iron intake of non-anæmic persons was 11.4 milligrams, and that of anæmic women 10.0 milligrams; the highest intake of any of their subjects was below 23.2 milligrams, and only 7 out of 49 had more than 15 milligrams, yet the modal and mean hæmoglobin of these women were both much higher than those of the coolie woman in Assam.

Calcium is credited with iron-saving properties, a low calcium intake necessitating a high iron intake to prevent the occurrence of anæmia. The model diet given by Napier and Das Gupta (1937) to one series of cases contained an allowance of 456 grammes of milk a day which alone contributed 0.55 grammes calcium, but did not result in any improvement in the blood picture. Two efforts were made to test the therapeutic effect of calcium, given in the form of calcium lactate with cod-liver oil, one on a series of apparently normal women, and one on a series of sufferers from serious anæmia.

Three groups of women with hæmoglobin values ranging from 71 to 80 per cent were selected; the first received 30 grains of the citrate of iron and ammonium daily for two weeks; the second received 20 grains of calcium lactate, with cod-liver oil, daily, and the third received both the iron and the calcium for the same period. The hæmoglobin values were again estimated at the end of the third week. The changes are set out in Table XI from which it will be seen that the calcium did not produce any increase in the hæmoglobin level.

The decreases in the means of the first two groups are not statistically significant, and the lack of improvement in those under iron treatment is only to be expected where these small doses are used.

TABLE XI.

The mean hæmoglobin values of three groups of women, before and after treatment with (1) 30 grains of citrate of iron and ammonium daily for two weeks, (2) 20 grains calcium lactate for two weeks, and (3) both the iron and calcium.

Group.	Drug.	Number in group.	Mean Hb percentage at beginning.	Mean Hb percentage after treatment.
1	Iron only ..	12	75.5	72.9
2	Calcium only ..	11	74.5	73.3
3	Iron and calcium ..	12	74.2	73.8

Hb taken on the Hellige scale, on which 100 per cent is equal to 13.67 grammes Hb per 100 c.c. blood.

The experiment on severely anæmic women involved the addition of calcium lactate to the normal iron treatment. It was abandoned shortly after the start, as it soon became obvious that there was no therapeutic advantage, and a suspicion

arose in the mind of the author that it might even have a harmful effect ; whatever the theoretical advantages of a continuation of the experiment, it was not considered justifiable to do so once this suspicion had formed. A consideration of the negative results of the experiment on healthy women, and of the dietetic experiment of Napier and Das Gupta, makes it highly improbable that the anæmia can be improved by the addition of calcium in any form to the food.

The amount of carotene and vitamin A in the diets is much below the standard of 1,200 International units quoted by Aykroyd, a finding in agreement with the observed frequency of symptoms of deficiency of this substance, but there does not appear to be any good reason to connect this shortage with the presence of anæmia. The other vitamin of which there is some possible deficiency, though only a slight one, is vitamin C. With the one exception already mentioned no suggestion of scurvy has been seen amongst the population, and the clinical and pathological pictures do not correspond with those attributed to shortage of vitamin C. There does not, therefore, seem to be any reason to connect the prevalence of anæmia with a shortage of either vitamin A or C, especially when the negative value of Napier and Das Gupta's diet, which contained an adequate supply of both these substances, is borne in mind.

The vitamin-B complex is present in adequate amounts in the diet, and no symptoms suggestive of a shortage of it have ever been noted. Although substances rich in vitamin D are absent from the diet, their absence is apparently compensated for by the normally brilliant sun.

Although these diets fall far short of all ideal standards which have been suggested, there does not appear to be any sound reason resulting from an examination of the analyses to suggest that their deficiencies cause anæmia in those who consume them.

THE HÆMOGLOBIN LEVELS ATTAINED BY PERSONS CONSUMING DIFFERENT VARIETIES OF RICE.

In searching for an explanation for the difference in hæmoglobin frequencies on different estates the possibility was considered that there might be some variation in the diets consumed. A superficial review indicated no such differences, but a more thorough examination revealed that the variety of rice most commonly consumed varied from estate to estate, and a possible connection between the consumption of one or other of these varieties and the prevalence of anæmia was therefore sought.

The term 'rice' has previously been used unqualified. Four varieties which differ in appearance, taste, analysis, and in that vague entity 'protective value' are normally consumed. Rice may be milled or home pounded. Milled rice normally, though not necessarily, is almost devoid of pericarp and germ. Home-pounded rice retains a considerable quantity of pericarp, and most of the germ. Before it is milled or pounded, the unhusked rice may be heated in hot water or steam, when its characters are altered and it is termed 'parboiled' ; in other cases it is used 'raw'. The four varieties of rice are thus, milled parboiled, milled raw, home-pounded parboiled, and home-pounded raw.

In the census of 1936-37, every person was asked which variety of rice he or she normally consumed. There is some seasonable variation in many people's habits, the number consuming home-pounded rice being greatest immediately after

the rice crop is harvested, and slowly declining thereafter. In making inquiries emphasis was laid on the normal habits of the subject for the greater part of the year, and not the variety consumed at that particular time.

With the information obtained it was possible to divide the population into four groups, each consuming one brand of rice, and to assign to each group its own hæmoglobin frequency distribution. This was done separately for men, women, and children, the results for women being reproduced in a summarized form in Table XII:—

TABLE XII.

A summarized frequency distribution of hæmoglobin values of women, classified according to the variety of rice normally consumed. Hæmoglobin estimated by the Tallqvist scale.

Rice consumed.	Number in group.	PERCENTAGE OF TOTAL WITH A HÆMOGLOBIN VALUE OF:—			
		50 or less.	55 to 60.	65 to 70.	75 and over.
Milled raw ..	443	15·3	27·1	40·2	17·4
Milled parboiled ..	237	14·3	22·3	44·3	19·1
Home-pounded raw ..	518	12·4	25·1	47·7	14·8
Home-pounded parboiled	1,465	7·6	17·7	53·0	21·7

The table reveals a very much lower incidence of both severe and moderate anæmia amongst those women consuming home-pounded parboiled rice than amongst those consuming the other three varieties. The hypothesis that this difference is due merely to the error of random sampling is made an extremely unlikely one by analysis by Pearson's χ^2 test. If the four groups were in fact identical, then the chances against the production by pure chance of a group so widely differing from the others as does the fourth group would be over three hundred thousand to one ($\chi^2 = 28·34$, $P = 0·00000332$).

The findings in men and in children corresponded in nature with those set out for women, though the lesser amount of anæmia in these two groups made the differences less marked. There can be no reasonable doubt that the incidence of anæmia is significantly less amongst those persons who normally consume home-pounded parboiled rice than in those consuming the three other varieties.

This demonstration of a relationship, however definite, does not prove that the relationship is a causal one. A third factor might influence the hæmoglobin level, and at the same time be associated with the normal eating habits, and until this possibility is excluded it would be unwise to assume that the diet has a direct effect on the hæmoglobin level.

There are many such possibilities of fallacy. It has already been shown that the incidence of anæmia varies from estate to estate, and that the variety of rice consumed by the majority also varies. The choice between home-pounded and milled rice depends, amongst other things, on the availability of rice, economic conditions, and the proportion of the population who have settled down to cultivate their own land. The choice between raw and parboiled rice depends largely on

upbringing, and in the case of milled rice also on availability. In various direct and indirect ways economic conditions become linked with the varieties of rice normally taken; the marked linkage is best illustrated by the fact that a very high proportion of widows consume milled parboiled rice.

There are two methods by which a causal relationship could be demonstrated; by demonstrating a relationship between change in dietary habits, and change in hæmoglobin level; and a demonstration that the association remained after all related factors had been eliminated. The first course was not possible to the author; the second was therefore attempted.

AN EXACT COMPARISON OF THE HÆMOGLOBIN LEVELS ASSOCIATED WITH CONSUMPTION OF FOUR VARIETIES OF RICE.

An experiment was designed with the aim of securing one hundred groups of women, in each of which there were to be four women, one consuming each variety of rice, but otherwise resembling each other as closely as possible in (1) age; (2) 'civil state', single, married, or widowed; (3) number of dependent children; (4) period of residence in Assam; and (5) the estate on which they lived and the company which employed them. It was thought that if these similarities could be secured, associated factors depending on economic conditions, age, surroundings, exposure to disease, and immunity to disease, could be ignored, and any remaining relationship between diet and hæmoglobin value considered as a causal one.

In this experiment all examinations were, without exception, carried out by the author, and the Hellige hæmoglobin estimation technique was used. Important errors of observation are, therefore, believed to have been eliminated.

The experiment was confined to adult women in order to exclude sex difference and the more pronounced age differences. Women who had been resident in Assam for less than three years were excluded, in order to avoid confusion from possibly undiscovered cases of self-privation.

The most important part of the work was the selection of the groups of women. A key woman for each group was chosen by noting the appropriate particulars of every woman eating milled parboiled rice, the variety least commonly consumed. The census lists were then searched, starting from the point where the key woman was found, and the next woman on the list, consuming another brand of rice, who resembled the key woman within the following limits was selected: (1) age within ten years; (2) the same 'civil state', single, married, or widowed; (3) not more than one more or less dependent children; (4) the period of residence in Assam to fall into the same group, 3 to 6 years, 6 to 10 years, 10 to 20 years, over 20 years, or born in Assam; and (5) to be employed by the same company, and to live in a group of lines reasonably near to the key woman. This process was continued until the group of four was complete.

The selections were made in a completely unbiased manner, and Hellige estimations were only made after the group of four had been completed. It was not possible to arrange one hundred groups, as had been planned, but 69 groups were secured. It is believed that in each group the four women were as nearly as possible identical in all relevant ways except the variety of rice normally consumed.

The results of the hæmoglobin estimations of these 69 groups of four women are set out in Table XIII. in the form of a frequency table, corresponding in form to

TABLE XIII.

The frequency distribution of hæmoglobin values in four groups of 69 women, each of which was made up, of consumers of different varieties of rice.

Series.	PERCENTAGE OF TOTAL WITH A HÆMOGLOBIN VALUE OF:—														Mean.	Standard deviation.
	Below 40	40 to 44	45 to 49	50 to 54	55 to 59	60 to 64	65 to 69	70 to 74	75 to 79	80 to 84	85 to 89	90 to 94	95 to 99	100 and over.	Number 50 or below.	
Milled raw ..	2 (35, 38)	3	1	3	3	9	5	17	10	7	7	2	6	70.77 12.95
Milled parboiled	2 (24, 39)	1	4	5	4	9	6	12	13	6	4	1	1	1	9	67.10 13.87
Home-pounded raw.	3 (16, 37, 39)	..	2	4	3	4	7	15	11	9	2	8	1	..	6	71.72 14.61
Home-pounded parboiled.	1 (34)	..	1	2	6	6	8	6	15	6	12	5	1	..	2	75.93 12.46

Hæmoglobin estimated by the Hellige instrument, on which 100 per cent = 13.67 grammes Hb per 100 c.c. blood.
Figures in brackets indicate actual hæmoglobin values.

that used in discussing the result of the Tallqvist estimations; the results are essentially the same, the average hæmoglobin and the proportion of each group severely anæmic fall into the same order as in the case of the Tallqvist series.

In the present series the average hæmoglobin of the groups are statistically significant figures, and they are compared in Table XIV. The significance of each difference between a pair of observations is expressed in terms of the odds against such a difference being found by chance as the result of the error of random sampling, which has been calculated by Pearson's χ^2 test.

TABLE XIV.

A comparison of the mean hæmoglobin of four groups of women, resembling each other except in that each group were consumers of a different variety of rice.

Types of rice compared.			Mean Hb of con- sumers, per cent.	Difference, per cent.	Standard error of difference.	Probability that the difference is real and not due to chance.
1	{ Home-pounded parboiled .. Home-pounded raw	75.93 71.72	4.21	2.31	14 : 1
2	{ Home-pounded parboiled .. Milled raw	75.93 70.77			
3	{ Home-pounded parboiled .. Milled parboiled	75.93 67.10	8.83	2.27	8,820 : 1
4	{ Home-pounded raw .. Milled parboiled	71.72 67.10			
5	{ Home-pounded raw .. Milled raw	71.72 70.77	0.95	2.38	0.15 : 1
6	{ Milled parboiled .. Milled raw	67.10 70.77			
7	{ Combined home-pounded .. Combined milled	73.82 68.93	4.89	1.63	370 : 1

Hæmoglobin estimated by Hellige technique, 100 per cent = 13.67 grammes Hb per 100 c.c. blood.

It is apparent from Table XIV that home-pounded parboiled rice is greatly superior in hæmogenic properties to milled parboiled rice, and to a lesser extent superior to milled raw rice (*see* comparisons 3 and 2). Milled raw rice and home-pounded raw rice occupy an intermediate position in the scale between the two types of parboiled rice ; there is, therefore, no clear distinction which demonstrates that either milling or parboiling has a harmful effect, though the analysis leaves no doubt that a combination of the two produces a rice very poor in hæmogenic power, and that the richest in this power is the home-pounded parboiled product.

The explanation of these differences is not clear, and little information is obtained by a study of the analyses of the four varieties given by Aykroyd (*loc. cit.*). In his tables home-pounded parboiled rice is stated to be richer than any other variety in calcium, phosphorus, and iron. In none of these is the milled parboiled rice inferior to milled raw rice, whereas there is a definite suggestion that it is inferior in hæmogenic power. Despite the lack of clear explanation, the presence of a very clear difference in the hæmogenic powers of these types of rice is an undeniable fact. The care which was taken to avoid confusing factors, and to make the four groups of women practically identical makes it almost certain that the relationship is a causal one.

Both the Tallqvist and the Hellige series show a difference between these types, and the Hellige series makes it highly probable that the connection is a direct one. The Hellige series also, however, makes it highly probable that this dietary factor is a subsidiary one in the production of anæmia, and not the main cause. If the main cause were some factor dependent on these brands of rice, the consumers of the most favourable variety should show not only a significantly higher mean hæmoglobin, but also a significantly more compact grouping round their own mean, which would be shown by a standard deviation markedly less than in other series. The standard deviations in these series are not significantly different from each other. The consumers of home-pounded raw rice, though one rung higher up the ladder than the consumers of other varieties, are still prevented from reaching the top by some other factor which has not yet been disclosed.

Part V.

ANÆMIA AND MALARIA.

EPIDEMIOLOGICAL CONSIDERATIONS.

It has been noted, in discussing the incidence of anæmia, that there is a significant difference in the incidence on different estates. In searching for a cause of the anæmia, a search was made for some other factor which showed comparable variations on these estates. The severity of malaria seemed to be an unlikely connection, because the estate on which anæmia was, and for several years had been, least notable was one of the most malarious. On this estate only 3·1 per cent of women were severely anæmic at the last census, as compared with 10·4 per cent in the general population, and the spleen rate had been consistently over 70 per cent until 1936, when it was reduced to 52 per cent. On the other hand, the estate in which anæmia was most marked, with 20·2 per cent of severely anæmic women, had a spleen rate which varied from 35 to 26 per cent.

All possible factors which might conceivably be considered to have any bearing on resistance to disease, nutrition, exposure to risk of disease, or the efficacy of treatment were considered. With one exception no connection was found to exist between the factors examined and the incidence of anæmia.

One factor showed similar variations. On those estates which had a settled labour force and did not recruit short-term labour, anæmia was much less prevalent than on those taking large numbers of recruits. The intermediate estates had an intermediate incidence of anæmia, a point which is illustrated in Table XV:—

TABLE XV.

The relationship between the proportion of recently recruited population and the incidence of anæmia.

Estate.	Percentage of population resident for less than three years.	Percentage of women severely anæmic.
1*	Nil*	8·9
2	0·2	3·7
3	0·6	3·1
4	7·8	10·5
5	11·8	14·9
6	14·1	9·9
7	16·8	11·9
8	19·8	18·2
9	22·0	21·2
10	31·8	16·3
11	45·0	20·2

* On this estate, and this estate only, the lines are intermingled with a village with a floating population.

The co-efficient of correlation between these two groups of figures is 0.82, with a standard error of 0.1. These figures would at first give the impression that the certainty of their association was fantastically high, but the small number of groups involved makes the certainty less than it would appear. In the table the percentages of new residents have been arranged in ascending order, and the percentages of anæmic women show a corresponding general increase, though the order is not exactly that of increasing frequency. The three estates with least anæmia head the list, and the four with most anæmia come at the foot. The chances of this occurring by pure chance, had there in fact been no connection, are represented by the equation $(3/11)^3 \times (4/8)^4 = 0.001268$, or 789 to 1 against; a degree of certainty which is statistically accepted as very highly significant; there can be no reasonable doubt that these two variables are closely associated. The connection is the more strange in that it has been demonstrated (*see* Part III) that the incidence of anæmia amongst newly recruited labourers is not higher than that amongst settled labourers. It means that the mixture of the two groups in one population is associated with a deterioration in the health of both.

A possible explanation of this curious connection is suggested by the experiments of Greenwood and Topley (quoted by Greenwood, 1932). They showed that the continuation and severity of an experimentally induced epidemic in a herd of mice was greatly affected by the number of new entrants to the herd, and that in the presence of many new entrants the severity increased amongst old salted mice as well as amongst the new ones.

This is the only apparent analogy, and it clearly suggests the investigation of all infective causes which might influence the degree of anæmia; although malaria had been excluded as a probable cause on first review, it was thought best to include it in the second review. The first had considered the severity of malaria as varying only with the rate of transmission, of which the spleen rate is thought to give an indication; the second considered as a major possibility an upset of the balance between infection and the premunition which holds the obvious signs of the disease in check in a population which has been exposed to the disease for a long period.

The discovery of malaria parasites in the peripheral blood is only possible in one or two per cent of adult women, though there are very good reasons for believing that the infection rate is far higher than this, and the search for parasites does not offer much hope of throwing light on the subject. It appeared that there were two possible lines of approach, firstly by the treatment of anæmic women on the principle that they were in fact suffering from malaria, and secondly by a more thorough search for parasites. If the anæmia were due to a disturbance of the balance of immunity, then the first method should result in an improvement of the blood picture; the results of the second method, if the hypothesis is correct, are less surely predictable because it was not assumed that immunity was disturbed in every individual of the population, and the discovery of parasites by finer methods of examination would throw little light on the immune condition of the individual.

TREATMENT OF ANÆMIC WOMEN.

The intention in this experiment was to treat a representative sample of women, who showed that mild anæmia which is most common, on the hypothesis that they

were suffering from malaria. Those suffering from the more severe forms of clinical anæmia, for whom an efficient iron treatment is available, and in whom no other form of treatment is justifiable, were excluded. The women included were thus of the group which considered themselves quite healthy.

It was decided to use 'atebrin for injection' as the anti-malarial drug. It was not necessarily believed that atebrin for injection had any superior therapeutic powers compared with oral atebrin or quinine. The reason for choosing it was that it was the only available drug, and method of administration, which could be administered in full therapeutic doses to women who considered themselves not in need of treatment. It would have been very difficult to persuade them to take a long course of oral quinine or atebrin, and to be quite sure that it had been taken. There was no doubt that the atebrin for injection had been administered, and retained.

It appeared illogical to expect an improvement in hæmoglobin, even after the causative agent of the anæmia had been removed, unless some therapeutic iron was also administered, as possibly many months might be required for the ingestion in normal diet of the iron required for hæmoglobin regeneration. Small doses of iron were, therefore, given to every case treated, and an identical dosage of iron to an untreated control bracketed with each case.

Cases and controls were secured in a number of estate hospitals. A large number of women attending hospital as attendants on sick children, and also sufferers from minor surgical ailments, cuts, bruises, and sores, were examined. Those with a hæmoglobin over 80 per cent on the Hellige scale were rejected as not anæmic, and those with a hæmoglobin under 35 per cent rejected as being too anæmic to be subjected to experimental treatment. Women falling between these two levels were taken alternately, as cases and controls, and each case was bracketed with a particular control. The case received two injections of 0.3 g. atebrin with an interval of 24 hours between injections. The control received no such treatment. The citrate of iron and ammonium was then administered to both, the intention being to administer 30 grains daily for two weeks. When either the case or control failed to receive the dose, it was withheld from the partner. As a result all couples, case and control, received identical doses of iron, and the majority received their full treatment. After two months' interval from the date of injection, the hæmoglobin was again estimated. It was not considered desirable to make intermediate examinations, as the repeated drawing of blood, associated with injections, might have caused difficulty amongst the subjects.

No ill effects of any sort were noted in the 44 women receiving injections. On one estate the control women later demanded injections because there had been a marked subjective sense of improved well-being in those who had received injections, and they did not understand why they should be deprived of this advantage.

In this way 44 couples were secured. Two cases and three controls were later eliminated from the series for the following reasons: (1) a case died of intercurrent disease; (2) a case developed a severe sloughing carbuncle of the neck with the loss of several square inches of skin, hæmoglobin 65 per cent at the time of injection, 50 per cent at time of illness, 63 per cent at the end of two months; (3) a control

suffered from pneumonia, hæmoglobin 73 per cent at start, 48 per cent during illness; (4) and (5) two controls could not be traced at the time of the second examination.

The perfect bracketing of cases and controls was upset by this exclusion of five women. The unpaired five have been left in the series as they all took their complete iron course, and therefore approximately match each other. The final results would not be altered by their exclusion because the mean improvement shown by them was almost identical with the mean of the whole series. The mean improvement in each group is shown in Table XVI:—

TABLE XVI.

The effect on the hæmoglobin level of two injections of atabrin, given to 42 women, with a series of 41 control women who received no atabrin.

Series.	BEFORE TREATMENT.			AFTER TWO MONTHS.			DIFFERENCE BETWEEN 1 AND 4.	STANDARD ERROR OF DIFFERENCE.
	1 Mean Hb per cent.	2 Standard deviation.	3 Stan- dard error of mean.	4 Mean Hb per cent.	5 Standard deviation.	6 Standard error of mean.		
42 cases ..	63.55	10.8	1.67	70.98	11.7	1.80	7.43	2.46
41 controls ..	65.94	8.27	1.28	67.16	11.8	1.84	1.22	2.24

Hæmoglobin estimated by Hellige technique, 100 per cent = 13.67 grammes Hb per 100 c.c. blood.

There was a marked, but not significant, difference between the mean hæmoglobin of the two series as first recorded. The 'case' group was 2.39 per cent less than the 'control' group, a difference which might be expected to occur by pure chance once in four series. The standard deviation was also considerably greater in this group, the difference being such as might occur by chance once in about eleven trials. These differences are not of the order considered significant in statistical analysis.

After treatment there was an increase in the mean hæmoglobin level of the 'case' group of 7.43 per cent. An increase of this magnitude might only be expected as a chance result once in 228 times that the experiment was repeated. It is well outside the normal limits of error and is statistically highly significant.

The control series show no change in their mean hæmoglobin which might not be expected to occur by pure chance six times out of every ten that such a series was taken; the increase of 1.2 per cent is statistically quite negligible.

There thus appears to be strong reason for believing that the atebirin had a distinct effect on the hæmoglobin level. This impression is heightened by a more detailed analysis of the results.

In the treated group, 32 women increased their hæmoglobin, one remained unchanged, and 9 decreased. The mean increase amongst those who improved was 10·4 per cent, the mean decrease of those who deteriorated 3·8 per cent. The greatest increase was 36 per cent, and the greatest decrease 8 per cent.

In the control group, in 20 women the hæmoglobin increased, in 2 it remained unchanged, and in 19 it decreased. The mean increase was 8·7 per cent amongst those who improved; amongst those who deteriorated the mean decrease was 7·2 per cent. The greatest increase was 23 per cent, and the greatest decrease 16 per cent. Six women showed a decrease greater than the 8 per cent shown by the worst woman in the treated group.

TABLE XVII.

Hæmoglobin variations, classified according to the original level, in a group of women treated with atebirin, and a control group.

Original Hb per cent.	TREATED GROUP.				CONTROL GROUP.			
	Number.	Increased.	Unchanged.	Decreased.	Number.	Increased.	Unchanged.	Decreased.
35 to 39	1	1. (3)
40 to 44	2	2. (19, 3)
45 to 49	3	2. (6, 36)	..	1. (4)	3	2. (13, 6)	..	1. (3).
50 to 54	4	4. (19, 9, 8, 11)	1	1. (16).
55 to 59	2	2. (5, 1)	3	2. (13, 11)	..	1. (4).
60 to 64	5	4. (16, 8, 30, 7)	..	1. (4)	9	4. (23, 7, 7, 1)	1	4. (13, 10, 9, 3).
65 to 69	11	7. (18, 12, 12, 11, 9, 4, 3).	..	4. (8, 4, 3, 1)	8	4. (19, 12, 8, 5)	..	4. (19, 7, 6, 4).
70 to 74	7	6. (11, 11, 10, 9, 5, 1).	..	1. (3)	11	5. (15, 8, 4, 2, 3).	1	5. (8, 9, 5, 4, 3).
75 to 79	7	4. (14, 5, 4, 4)	1	2. (5, 2)	6	3. (12, 4, 2)	..	3. (4, 8, 11).

The figures in brackets indicate the actual change in hæmoglobin in the individual cases.

Hæmoglobin estimated by the Hellige technique, 100 per cent = 13·67 grammes Hb per 100 c.c. blood.

It is unfortunate that the method of random choice of cases and controls resulted in an uneven pair of series, with several more women with lower grades of hæmoglobin in the case group than in the control. Nevertheless the indications in Table XVII are clear. In the control group, whatever the original hæmoglobin, its subsequent alterations appear to have been a matter of pure chance. In the treated group the great majority of those with a hæmoglobin level below 65 per cent at the start of the experiment showed marked improvement. Of those with a hæmoglobin of 65 per cent or over at the start, 17 out of 25 showed improvement, one remained unchanged, and 7 showed a mean decrease of 3·7 per cent.

The possibility must be considered that, apart from its action on malaria parasites, atebriin is in itself a stimulant to hæmopoiesis. The scientific staff of the manufacturers are unaware of any such action although extensive investigations into its pharmacology have been made, during which such a point could hardly have been overlooked. There are very few records of observation on patients available. Vardey (1935) who described the changes following atebriin treatment in malaria cases did not note any change in a non-malarial control. Jarvis (1932) noted an average increase of 9·2 per cent in the hæmoglobin of five malaria cases during treatment with oral atebriin, an increase which appears to have continued after the end of treatment, and Duncan (1933) also treating patients suffering from malaria found an increase during treatment of a 6 per cent, which continued after the end of treatment. He attributed this increase to a combination of the effects of atebriin and rest and nourishment. The similarity between their increases and those found in the present series is notable.

It is, therefore, clearly demonstrated that atebriin by injection produced a significant increase in the hæmoglobin of these women, and there is reason to believe that such an increase might be due to the cure of undeclared malaria.

THE RELATION BETWEEN INFECTION OF THE PLACENTA WITH MALARIA PARASITES AND HÆMOGLOBIN LEVEL.

The most hopeful line along which a more thorough search for malaria parasites could be made appeared to be the examination of placental blood films. Blacklock and Gordon (1925*a, b*) made a thorough study of malaria in Freetown, Sierra Leone, by this method, and were able to demonstrate parasites in the placental films of 36 per cent of women in whom the parasite rate in the peripheral blood was 7·7 per cent. The present author had the advantage of studying their technique, and examining their specimens, and it was decided to use this method in studying the anæmias. It is true that the anæmia encountered in these women would normally be classified as 'anæmia of pregnancy', but as there had appeared little difference between this type and the usual anæmia, except that the former was more severe and more resistant to treatment, this was considered of little account.

Placental blood films were prepared by Assistant Medical Officers from a series of parturient cases, and the hæmoglobin was estimated by the author by the Hellige technique. Practical difficulties made the estimation of hæmoglobin immediately

after labour almost impossible, and there was the added disadvantage that, taken at that time, it might reflect the degree of hæmorrhage following labour more than the general blood picture of the patient. Estimations were therefore made after an interval which varied from seven to fourteen days. Previous experience with some thousands of Tallqvist estimations suggested that little change might be expected in the blood picture between the last stages of pregnancy and this stage of the puerperium.

A total of 85 blood films were secured, of which 4 were subsequently rejected from the series, two being too decomposed for examination, and in two cases it being not practicable to estimate the hæmoglobin. One case was included in which the sole hæmoglobin estimation was by the Tallqvist technique, two days before delivery. In this case the hæmoglobin was 35 per cent, and the patient died of heart failure immediately after delivery.

Of the remaining 81 films 9, or 11·1 per cent, were found to contain malaria parasites. In all cases the species was diagnosed as *Plasmodium falciparum*; in 6 the sporulating forms, seen by Blacklock and Gordon in all their cases, were present, in the other 3 ring forms only.

The hæmoglobin findings, together with their standard deviations and the standard errors of the means, are set out in Tables XVIII and XIX. The mean of the infected series was considerably greater than that of the uninfected, but the small number of cases in the former makes the difference statistically insignificant, being of the order such as might be expected to occur by pure chance once in every eleven times that the series was repeated.

TABLE XVIII.

The mean hæmoglobin in 9 women in whom malaria parasites were present in the placental blood, and in 72 in whom they were not found.

Series.	Mean hæmoglobin.	Standard deviation.	Standard error of mean.
Infected ..	69·4	16·3	3·8
Not infected ..	62·5	17·5	1·5

Hæmoglobin estimated by Hellige technique, 100 per cent = 13·67 grammes Hb per 100 c.c. blood.

TABLE XIX.

The frequency distribution of hæmoglobin values in two series of women, in one of which malaria parasites were present in the placental blood, and in one of which they were not found.

Hæmoglobin percentage value.	Distribution of uninfected series.	Distribution of infected series.
20 to 29 ..	2	<i>Nil.</i>
30 to 39 ..	6	1
40 to 49 ..	12	<i>Nil.</i>
50 to 59 ..	18	2
60 to 69 ..	9	1
70 to 79 ..	19	1
80 to 89 ..	11	4
90 to 99 ..	2	<i>Nil.</i>
100 and over ..	2	<i>Nil.</i>

Hæmoglobin estimated by Hellige technique, 100 per cent = 13·67 grammes Hb per 100 c.c. blood.

These observations give a quite clear answer to the question which it was intended to answer; the presence of malaria parasites in the placenta is not necessarily associated with a low hæmoglobin level. They suggest that parasites may tend to be associated with a hæmoglobin level rather above that in those in whom parasites were not found, but this suggestion is not of sufficient strength to be considered important.

The relationship between anæmia and malaria, therefore, remains uncertain. It has been shown that there is without doubt a relationship between the incidence of anæmia and the proportion of new entrants in the population, and analogy suggests an infective cause. The improvement in the blood condition that results from administration of atabrin suggests strongly that this infective cause is malaria, and though this hypothesis is not supported by the placental examinations, it is not thereby made improbable. There is another condition, blackwater fever, characterized by hæmolysis and anæmia, and now universally attributed to malaria, occurring only when non-immune persons mingle with a highly infected population, and in which the presence of malaria parasites is very difficult to demonstrate. The author believes it highly probable that further research will demonstrate a close connection between these two conditions.

CONCLUSIONS.

The work of Napier and Das Gupta has shown that there is some connection between the prevalent anæmia and infection with hookworm; it is the opinion of the author that it has also demonstrated the existence of some other factor, of greater importance than hookworm infection, which controls the prevalence of the disease, though hookworm infection may aggravate the severity, and make cure somewhat more difficult. A dietetic factor, depending on the variety of rice normally consumed, has been clearly proved in this work. This factor, though its importance is more clearly proved than is that of hookworm infection, is of the same order. It is not the predominant cause. It has been proved without reasonable doubt that a most important cause, probably the chief cause, is the mingling of newly arrived labourers with settled residents. The exact mechanism by which this mingling produces its effects is not certain; analogy strongly suggests that it operates through an infective process, the improvement of the anæmia by atabrin suggests that this process is malaria, but this is not supported by examination of placental films for malaria parasites.

It has not been possible for the author to complete the investigation to determine the mechanism by which the effect is produced. He would like to suggest to other workers, for proof or disproof, that it operates either by an admixture of different strains of malaria parasites, or by an upset in the balance between transmission and immunity which occurs when non-immune entrants mingle with an immune, or premune, population. Useful information might be gained from a comparative study of the blood pictures of similar groups of people living in malarious areas, and in areas entirely free from malaria.

Whatever the method of operation, one point of value in the prevention of the condition is clear; new entrants should not live in close contact with old settled inhabitants. It is the usual practice on estates to have the lines arranged in two or more distinct groups, often separated by considerable distances. So far as possible newly recruited labourers should be drafted to separate groups of lines in an effort to secure the segregation of the two classes of people. If this is done a marked reduction in the incidence of anæmia is to be expected.

A point of value in the treatment of the condition is also clear. The expectation of improvement in these cases may be increased by the administration of atabrin, together with the usual iron treatment.

Should the connection between anæmia and malaria which has been suggested prove to be a definite one, the indication of the increased importance of malaria control is also clear.

SUMMARY.

A very common condition of anæmia affecting estate labourers in Assam is described, and a brief account of the pathology, taken from the work of Napier and Das Gupta, given.

Doubt is expressed that the commonly accepted cause, hookworm infection, is in fact the chief cause of the condition. The doubt is based on the lack of correlation between the degree of infestation and the severity of anæmia, and on the uncertain effects of infestation on cure of the anæmia.

An attempt has been made to find other possible causes by means of an epidemiological study of the condition, and of the background on which it occurs. The technique of the various methods used is described.

An account is given of the district, and of the general characters of the population studied. The chief vital statistics and the death rates by age groups and causes are given, and also an account, numerical as far as possible, of the incidence of the chief diseases.

The frequency distribution of hæmoglobin findings in 9,042 persons, classified into men, women, and children, taken by the Tallqvist method is given. Of women 10.42 per cent, of children 5.33 per cent, and of men 2.77 per cent are classified as severely anæmic.

A comparison is made between the incidence of anæmia in newly recruited and settled women, and found to be approximately equal in the two groups. This is contrary to normally accepted belief.

A comparison is made of the incidence of anæmia on different estates, and marked differences demonstrated; this is also contrary to commonly expressed opinion.

The morbidity and mortality resulting from anæmia is estimated as being of the order of a loss of 3,000 working days and six lives per 1,000 persons per annum.

The diets normally consumed are described and analysed. They show little variation in constitution from day to day, consisting mainly of rice and a pulse. Fat is very deficient; the calcium intake is very low; the P : Ca ratio is high; iron intake is above the normal standards; carotene and vitamin A are very deficient, a finding which corresponds with clinical findings; other vitamin standards are nearly attained.

An experiment to determine any possible therapeutic effect of the oral administration of calcium gave negative results.

An analysis of the Tallqvist readings of the 9,042 persons examined, and for each of whom the variety of rice normally consumed was known, showed a very significantly lower incidence of severe anæmia amongst those consuming home-pounded parboiled rice than amongst those consuming other varieties.

An exact comparison of the hæmogenic properties of the four varieties of rice normally consumed was made. Sixty-nine groups of four women were examined;

in each group the four women closely resembled each other in all factors likely to influence the hæmoglobin level, except that each consumed a different brand of rice, one consuming each of the following: home-pounded parboiled; home-pounded raw; milled parboiled; milled raw. The mean hæmoglobin, estimated by the Hellige technique, of those consuming different varieties was then compared.

The mean hæmoglobin value of the series were: home-pounded parboiled, 75·93 per cent; home-pounded raw, 71·72 per cent; milled raw, 70·77 per cent; milled parboiled, 67·10 per cent.

The difference between the home-pounded parboiled and milled parboiled series is very highly significant; that between the home-pounded parboiled and milled raw series is also significant.

The explanation of the differences is not clear from a study of the analyses of the four rices, but is definite. It is considered that the varying hæmogenic properties of the rices is a subsidiary, and not the predominant, cause of the prevailing anæmia.

The varying incidence of anæmia on different estates was studied, and one significant associated factor disclosed. Although the incidence of anæmia amongst newly recruited and settled labourers on any estate is about equal, yet anæmia is significantly most frequent, amongst both types of labourers, on estates where there is a high proportion of newly recruited labour.

An analogy is suggested with Greenwood and Topley's experimental epidemics, and the possibility of an infective cause is, therefore, suggested.

Malaria, as estimated by the spleen rate, shows no correlation with the incidence of anæmia, but it is considered as a possible cause, on the hypothetical grounds that the mixture of new and settled persons with varying degrees of immunity might cause an upset of the balance between infection and immunity.

Two experiments were designed to test this hypothesis; in the first, 42 mildly anæmic women were treated with atebirin by injection, and 41 control women left untreated, and their hæmoglobin changes watched; in the second, 81 placental films were examined for malaria parasites, and the findings correlated with the hæmoglobin findings. There was a highly significant increase in the hæmoglobin of the 42 treated women and none in that of the controls. The second experiment revealed no significant difference between the positive and negative cases.

The investigation had to be stopped at this stage, but the possible implications of the last findings are discussed.

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ON M AND N IN BLOOD GROUPS: THE TECHNIQUE
OF TYPING, THE ANTIFLUIDS, FINDINGS
IN 300 INDIANS, AND ASSOCIATED
CONSIDERATIONS.

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INTRODUCTION.

THIS work was undertaken with a view to incorporating an important technique of forensic value with the routine of this laboratory.

Attempts were made to obtain the anti-M and the anti-N fluid for the preliminary typing of some of the professional donors under control of this laboratory, from a medico-legal worker in England. The latter's colleague expressed his regret at not being able to supply the fluids 'in the present state' of their work but recommended a commercial continental firm which he thought would supply satisfactory antifluids for the preliminary typing of bloods, preparatory to injecting rabbits for the preparation of the antifluids locally.

The antifluids were obtained from the aforesaid commercial firm. They were rather expensive; but the enclosed directions disclosed a very valuable technique. Unlike the procedure recommended by Wiener (1935) in America and by Harley (1936) in England the reaction could be performed macroscopically and quickly, exactly as ordinary macroscopic grouping tests are done.

Our next step was to prepare the antifluids acting briskly and specifically like the antifluids of the commercial continental firm. With the exercise of the usual care needed in quantitative absorption not much difficulty was encountered, only some antifluids had to be rejected. From the first batch of rabbits injected with

OM and ON r.b.c. we obtained the two antifuuids which acted briskly and specifically. The results of the unknown bloods tested with the commercial fluids and our own fluids were identical.

In this communication will be dealt with :—

- I. The technique of typing the r.b.c. of a subject,
- II. the technique of preparing the antifuuids for typing, and
- III. findings in 300 bloods from Indian cases in hospitals in Calcutta.
- IV. associated considerations.

Incidentally, the nomenclature I, II, III, and IV is now not only archaic but also incongruous with M, N, and MN. The nomenclature O, A, B, and AB has been used in this communication. A diagram illustrating the iso-hæmagglutinogens and the iso-hæmagglutinins is appended. With reference to the diagram the sub-groups with A₁ and A₂ and the distribution of M and N are explained, and equivalents in I, II, III, and IV are also given, for the benefit of those only familiar with the older nomenclature.

The human r.b.c. are now grouped by means of group specific sera and typed by means of type specific antifuuids.

I. THE TECHNIQUE OF TYPING THE R.B.C.

1. *Apparatus required.*

(i) Standard drill and wire gauge of the L. S. Starrett Co., U. S. A. Small capillary pipettes with lower ends fitting hole No. 58 are made with this gauge. Only the drawn part of a glass tubing, measuring 4 to 8 inches, is used. The upper ends are cut clean. The pipettes are worked with the finger tip. It is surprising how easily they draw up fluid with a suitable tilt of the container. When held vertically and allowed to deliver a drop in about a second they deliver 50 drops to a c.c. They are calibrated capillary finger pipettes.

Over fifty of such pipettes can be made from one foot of glass tubing $\frac{1}{2}$ inch in diameter. Some of the small ones are sterilized in plugged tubes. They are used in drawing the antifuuids from their phials. Others are used without further treatment.

Wire gauges of other makes and calibrations may be used.

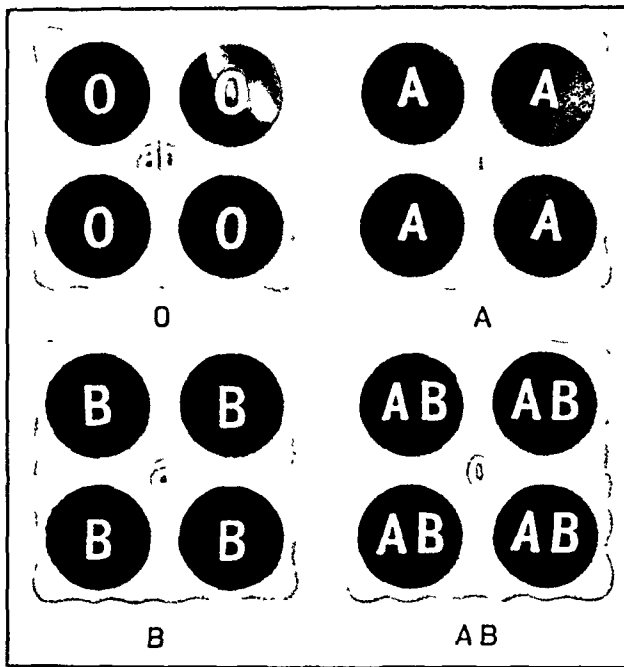
(ii) Glass slides, preferably old. On such slides drops of r.b.c. suspensions and the group testing serum or the antifuuids spread easily.

The obverse of each slide is divided into two halves by a line drawn with a grease pencil. The reverse is marked along the lower border with the distinguishing number of the specimen. The left half of the slide will receive either the group specific serum *a* or the anti-M fluid and the right half either the group specific serum *b* or the anti-N fluid.

(iii) Glass rods, about 5 inches long, with rounded ends, to stir and spread r.b.c. and antifuuids on the slides. One rod will stir the two halves of a slide.

(iv) Medium-sized Petri-dishes and small watch-glasses to make moist chambers. A piece of filter-paper is folded, soaked in water, and left in the watch-glass in the Petri-dish. Four slides can be conveniently placed, manipulated and inspected at stated intervals, in one dish.

PLATE XII.
Blood groups.



Jansky	Moss	New
I	IV	O
II	II	A
III	III	B
IV	I	AB

A and B are the iso-haemagglutinogens.

a and b are the corresponding iso-haemagglutinins.

The group is named after the iso-haemagglutinogens.

The four groups indicate the only four possibilities, compatible with life, in which the iso-haemagglutinogens and the iso-haemagglutinins can co-exist in the same subject. In a 'defective' group an iso-haemagglutinin which can exist compatibly with life is absent (e.g., O, a ; A, o).

Further division of A into A₁ and A₂ (and into A₁B and A₂B) increases the number of groups to six.

Quite unrelated to A and B are the haemagglutinogens (not iso-haemagglutinogens) M and N. They occur as M, N, or MN in all subjects of all groups. No subject is free from them. They differentiate three *types* in each *group* and thus make possible 12 descriptions of blood, if only the original four groups are considered, or 18 descriptions, if sub-groups with A₁ and A₂ are also considered. A blood may be:—

OM	ON	OMN.
AM	AN	AMN.
BM	BN	BMN.
ABM	ABN	ABMN.

There are difficulties of technique in determining A₁ and A₂. They are, therefore, ignored by many workers in forensic medicine. M and N are easily determinable and are receiving due recognition all over Europe and America.

of the same type as the recipient is desired, the compatibility must first be established by grouping.

3. *Antifluids.*

The anti-M and anti-N fluids obtained from the commercial firm were contained in 1 c.c. hermetically sealed ampoules. They were distinctly turbid, had a whitish deposit at the bottom, and smelt strongly of an antiseptic. Two ampoules, one of each antifluid, were shaken, opened and a calibrated capillary pipette dropped into each.

4. *Typing.*

Glass slides suitably divided and marked for each blood suspension were placed in the moist chamber. On the left of each slide was placed a drop from anti-M fluid and on the right a drop from anti-N fluid. Drops from the corresponding tubes of suspensions were added, mixed with stirring rods and spread over an area of about $\frac{3}{5}$ inch in diameter. Agglutination occurred almost immediately.

It was then decided to dilute the antifluids in the geometrical series $\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{8}$. . . until the agglutination occurred in 5 minutes. The weakest dilution capable of producing the agglutination, in an equal volume of the cell suspension, in 5 minutes, was taken as the minimal agglutinating dose. Twice the minimal agglutinating dose was employed in testing the suspension. It was found that the anti-M fluid had to be diluted with three volumes of normal saline containing 0.25 per cent tricesol and anti-N fluid with one volume only to give the required dose.

The reaction was recorded after 5 minutes, 15 minutes and 30 minutes. Some blood suspensions gave a \pm reaction in 5 minutes and a + reaction in 15 minutes. These bloods were re-tested with double the usual dose (2 drops of the dilution to one drop of the cell suspension) and a + reaction obtained in 5 minutes in most cases. In some cases the speed of the reaction could not be accelerated even with the undiluted antifluid. No new change occurred in a suspension after 15 minutes.

The slides were kept in the moist chamber all the time. The drops were set in motion, before taking the reading, by tilting and untilting the dish with a clockwise and counter-clockwise rotatory motion. Incipient agglutination was read with the aid of the hand lens. A mere sedimentation of the r.b.c. was at once differentiated from the agglutination by the cells arising from the slide and producing the appearance of a thin cloud dispersing before a gentle wind and forming patterns before going into a homogeneous suspension again. The preparations in the moist chamber resisted drying for 24 hours, the r.b.c. in most of them, however, were found lysed next day. No necessity was ever felt for removing a slide from the chamber for manipulation or inspection. Herein lies the superiority of the macroscopic technique over the microscopic technique. Manipulation and observation can never be made so easily and speedily under a microscope.

5. *Typing with the antifluids prepared by us.*

The antifluids prepared by us are found to correspond, in strength, to the diluted antifluids of the commercial firm. They are used undiluted and give in 5 minutes results which are identical with those obtained with the commercial antifluids, diluted and undiluted. The results are read in 5 minutes, 15 minutes, and 30 minutes as was done before. After 15 minutes no new change occurs, As was done before.

the blood suspensions which give a \pm reaction in 5 minutes are repeated with double the usual dose (2 drops of the undiluted antifluid of our own make and to one drop of the cell suspension) and a + reaction obtained in most cases. The results are recorded in the 4th and 5th columns of the above table.

II. THE TECHNIQUE OF PREPARING THE ANTIFLUIDS.

1. Collection of OM and ON bloods.

OM and ON bloods from professional donors are collected in Rous and Turner's solution, as recommended by Wiener (*loc. cit.*), as follows : 3·8 per cent sodium citrate solution, in lots of 20 c.c., is sterilized in 100-c.c. flasks ; 5·4 per cent glucose, in lots of 50 c.c., is sterilized in 250-c.c. flasks ; 30 c.c. of blood from a donor is drawn, added to the citrate solution, and the whole added to the glucose solution. The mixture is kept in a refrigerator. Fifteen c.c. of a packed deposit of r.b.c. can be obtained from the 100 c.c. of the mixture. Required quantities can be withdrawn with a 10 c.c. pipette. Strict sterility must be maintained. The mixture remains fit for use for over two months. A slight or even marked colour in the supernatant fluid can be ignored and so may be darkening of the deposit, as long as there is no smell and no turbidity or other signs of bacterial growth.

The mixture is centrifuged and the supernatant citrate and glucose solution removed. The deposit is washed once in sterile saline. These operations are done in rubber-capped centrifuge tubes when the r.b.c. are required for injection. For absorption the capping of the tube is not necessary.

2. Injection of rabbits for anti-OM and anti-ON sera and collection of the sera.

Full-grown rabbits weighing about 3 lb. (the usual weight of a rabbit purchased in Calcutta) are injected intravenously with a 50 per cent suspension of the r.b.c. as follows :—

				c.c.
1st injection	1
2nd injection, on the 4th day	1
3rd injection, on the 8th day	2
4th injection, on the 12th day	4

Ten days after the last injection the animals are bled. The serum is distributed in lots of 1·25 c.c. in small phials which are corked with paraffined corks, inactivated, and stored frozen (in the freezing chamber of a refrigerator).

3. Absorption of anti-OM and anti-ON sera with O and obtaining of the residual anti-M and anti-N fluids.

(i) *Absorption of anti-OM serum.*—So far we have found that a good serum when diluted 1 in 25* agglutinates both r.b.c. OM and ON in an equal volume of a 2 per cent suspension in 30 minutes. The clumps of the OM r.b.c. appear

* Two drops of serum, from the calibrated pipette, added to 1 c.c. of saline make a 2 in 50 dilution which is approximately a 1 in 25 dilution. Addition of an equal volume of saline gives a 1 in 50 dilution. Higher dilutions of 1 in 100 and 1 in 200 are obtained, if desired, by the same process of doubling the volume.

more compact in comparison with those of the ON cells. None of our sera has agglutinated the O r.b.c. in a 1 in 50 dilution.

One c.c. of the serum (or a little over, as much as can be removed from the phial) is added to 1 c.c. of a packed deposit of ON cells in a 15-c.c. centrifuge tube and the two mixed by gentle tapping and rotation. The r.b.c. are agglutinated into a gelatinous mass. The tube is left in an almost horizontal position in a moist chamber, for half an hour at room temperature, for half an hour in a refrigerator, and for half an hour again at room temperature. It is then centrifuged and a clear fluid (with or without a tinge of colour) measuring 0.1 c.c. to 0.2 c.c. more than the serum added (due to a tighter packing of the r.b.c.) separated.

One drop of the absorbed fluid is tested with one drop of a 2 per cent OM r.b.c. If agglutination commences in about two minutes and is complete in five minutes the potency is regarded as good.

One, two, and three drops of the absorbed fluid are next tested, on a slide marked into three compartments, with one drop of a 2 per cent ON r.b.c. If no agglutination occurs in any of the compartments in 30 minutes the specificity of the fluid is regarded as good.

If agglutination (definite or even a granularity) occurs with one, two, or three drops the specificity is defective. The serum must be re-absorbed with 1 c.c. to 0.5 c.c. (depending upon the degree of the agglutination) of packed ON r.b.c. The re-absorption does not lower the titre for OM r.b.c.

The absorbed fluid of a good potency and specificity is the anti-M fluid. It is collected with a capillary test pipette, transferred to a phial, and preserved with 0.25 per cent tricresol.* One c.c. of the fluid suffices for 40 to 50 tests.

(ii) *Absorption of anti-ON serum.*—The serum when diluted 1 in 100 to 1 in 200 agglutinates both r.b.c. OM and ON in equal volume of a 2 per cent suspension. The ON r.b.c. are made more compact in comparison with the OM cells.

The serum is diluted 1 in 4 to 1 in 8 with normal saline, the rest of the procedure is the same as described for the absorption of the anti-OM serum, with OM substituted for ON.

The re-absorption of the anti-ON serum with OM r.b.c. lowers the titre for ON r.b.c. also. The finished anti-N fluid, however, acts as briskly as the anti-M fluid, the reason being that a 1 in 4 dilution of a serum found agglutinating perfectly in a 1 in 100 dilution and giving a \pm reaction in the next higher dilution is stronger than the pure serum which agglutinates in a 1 in 25 dilution but not in a 1 in 50 dilution. The discrepancy is due to the inequality of spacing in the geometrical series, 1/25, 1/50, 1/100, 1/200. . . .

Contrary to the published findings we have had very little difficulty in the preparation of the anti-N fluids. Our anti-ON sera have usually acted briskly, sharply, and in a higher dilution than the anti-OM sera; and our anti-N fluids have been of good potency and specificity. Our anti-OM sera, on the other hand, have often been poor and our anti-M fluids inert.

* For the purpose of adding tricresol volume of the antifluid is looked upon as 1 c.c. One drop of tricresol, from the calibrated pipette, added to 0.14 c.c. of ether = 1 in 8 of tricresol. A drop of the mixture added to 1 c.c. of the antifluid gives the desired 1 in 400 (8×50) or 0.25 per cent.

4. *Sera not conforming to the standard.*

It so happened that the first two lots of sera we collected yielded antifuuids of good potency and specificity when treated according to the procedure described above. Later, we found that there were other lots of sera which behaved differently. Amongst anti-OM sera, some had a titre lower than 1/25 for O and yielded weak anti-M fluids; they were of course useless; others had a low titre for O but yielded a satisfactory anti-M fluid. Amongst anti-ON sera some had a low titre for O and yielded an inert fluid against N; others had a high titre for O yet yielded an equally inert fluid against N. Some animals bled by cardio puncture yielded satisfactory anti-OM and anti-ON sera and satisfactory anti-M and anti-N fluids; a month later it was found that a single intraperitoneal injection raised the titre against O to almost the original level yet the antifuuids obtained were worthless. The preparation of the antifuuids, therefore, involves considerable rejection and selection and is not as easy as the preparation of a hæmolytic amboceptor or an agglutinating anti-bacterial serum. We have not, however, found it 'notoriously difficult' (Thomas, 1938); and we have not exhausted all devices yet.

We have called the unabsorbed products antisera and the finished products antifuuids. This seems to be a fairly well accepted nomenclature and should be adopted even if it is disregarded in the writings of some workers.

5. *Stability of the antifuuids.*

Our antifuuids have been kept in the cold for several weeks (until exhausted) without deterioration. One lot was left at room temperature also for 10 days (1st to 10th October, the average maximum temperature 87°F.) and showed no deterioration.

The antisera are known to be very stable.

III. FINDINGS IN 300 BLOODS FROM INDIAN CASES IN HOSPITALS IN CALCUTTA.

The following table gives the group and the type of 300 Indians, in Calcutta hospitals, taken serially:—

Groups.	TYPES.		
	M	N	MN
Per cent	Per cent	Per cent	Per cent
O 80 (26·7)	33 (41·2)	10 (12·5)	37 (46·2)
A 80 (26·7)	31 (38·7)	11 (13·7)	38 (47·5)
B 113 (37·7)	51 (45·1)	11 (9·7)	51 (45·1)
AB 27 (9)	13 (48·1)	0	14 (51·8)
TOTALS 300	128 (42·7)	32 (10·7)	140 (46·7)

The following statistical tests have been applied to the data by Dr. R. B. Lal, Professor of Vital Statistics and Officiating Director, the All-India Institute of Hygiene and Public Health, Calcutta :—

(i) *Re. groups.*—If p , q , and r represent the proportional frequencies of the genes A, B, and R respectively, then according to the theory the values of p , q , and r may be derived with the help of the following relations :—

Phenotype.	Proportional frequencies.
\bar{O}	r^2
\bar{A}	$p^2 + 2pr$
B	$q^2 + 2qr$
$\bar{A}\bar{B}$	$2pq$

which give rise to equations

$$\begin{aligned} p &= 1 - \sqrt{\bar{O} + \bar{B}} \\ q &= 1 - \sqrt{\bar{O} + \bar{A}} \\ r &= \sqrt{\bar{O}}. \end{aligned}$$

The following values are obtained from the data :—

$$\begin{aligned} p &= 0.1979 \\ q &= 0.2697 \\ r &= 0.5164. \end{aligned}$$

These give $p + q + r = 0.9840$.

According to the theory of Bernstein, $p + q + r$ must equal unity. We have, therefore, to see whether or not the difference between 1 and 0.9840 is statistically significant. The formula of standard error of this difference has been given by Bernstein as :—

$$\sqrt{\frac{pq}{2N(1-p)(1-q)}} \quad \text{which gives } 0.0119$$

N is the total frequency.

The difference divided by this standard error of the difference is 1.34.

In this case, therefore, the recorded values do not differ from those expected on the theory.

(ii) *Re. types.*—If a , b , and c represent respectively the observed number of individuals of types M, MN, and N and T is the total number of observations, then

$$\begin{aligned} a &= 128, \\ b &= 140, \\ c &= 32, \\ T &= 300. \end{aligned}$$

In order to test the hypothesis of Landsteiner and Levine we calculate, on this hypothesis, the expected frequencies corresponding to a , b , and c . With the

help of the method of maximum likelihood it is seen that if a_0 , b_0 , and c_0 denote respectively the corresponding expected frequencies, then

$$a_0 = (2a + b)^2/4T = 130.68$$

$$b_0 = (2a + b)(2c + b)/2T = 134.64$$

$$c_0 = (2c + b)^2/4T = 34.68.$$

We have now to test whether or not the differences between the expected and the observed frequencies fall within the range of errors of sampling. For this purpose the value of χ^2 has been calculated from the formula:—

$$\chi^2 = \frac{(a - a_0)^2}{a_0} + \frac{(b - b_0)^2}{b_0} + \frac{(c - c_0)^2}{c_0} = 0.475.$$

This value shows that the recorded values of a , b , and c do not differ to any significant extent from the corresponding values expected from the theory.

Additional observations on the series are:—

- (i) In type MN, \bar{M} equalled \bar{N} only in ABMN on eight occasions. Otherwise \bar{M} was stronger than \bar{N} . \bar{N} was never stronger than \bar{M} .
- (ii) Both \bar{M} and \bar{N} were stronger when alone than when together.
- (iii) On seven occasions \bar{M} was rather weak: twice in OM, twice in AM and thrice in BM.
- (iv) On one occasion only was \bar{N} rather weak, in a BN.
- (v) An ABN was not found in the series.

It has been stated by other workers that the type does not change with the morbid states. Our hospital cases, therefore, are representative of the mixed Indian population of Calcutta.

Fifty bloods from European cases in hospitals in Calcutta were also grouped and typed, more with a view to testing our antifuuids than confirming known findings. Our findings are:—

Groups.	TYPES.		
	\bar{M}	\bar{N}	MN
O 19 ..	6	3	10
A 19 ..	6	3	10
B 11 ..	3	3	5
AB 1 ..	0	0	1
TOTALS 50 ..	15	9	26

Additional observations on the small series are :—

- (i) In type MN, M equalled N on two occasions (AMN and ABMN). Only on one occasion was N stronger than M. Otherwise M was always stronger than N.
- (ii) Both M and N were stronger when alone than when together.
- (iii) A weak M was not found.
- (iv) A rather weak N was found on three occasions, once in ON, once in OMN and once in AMN.
- (v) An ABN was not found.

The following is a comparison :—

Author.	Population.	Number tested.	FREQUENCIES OF TYPES (PERCENTAGE).		
			M	N	MN
Landsteiner and Levine* ..	<i>New York.</i>				
	Whites ..	532	26.1	20.3	53.6 (± 1.50)
	Coloured ..	181	27.6	24.9	47.5
	<i>American Indians.</i>				
		(i) 81	62.9	3.7	33.3
		(ii) 124	58.0	5.6	36.3
Schiff*	<i>Germans</i> ..	3,333	30.9	19.6	49.4
Crome*	<i>Germans</i> ..	1,300	32.5	18.5	49.0
Kossovitich* ..	<i>French</i> ..	400	33.0	21.2	45.8
Harley (<i>loc. cit.</i>) ..	<i>English</i> ..	200	32.5	19.5	48.5
Boyd and Boyd (1938) ..	<i>Rwala Beduin</i> ..	208	57.5	5.8	36.7
Present writers ..	<i>Calcutta.</i>				
	Indians ..	300	42.7	10.7	46.7
	Europeans ..	50	30.0	18.0	52.0

* Quoted from Wiener (*loc. cit.*, page 133). Only one figure after the decimal taken.

IV. ASSOCIATED CONSIDERATIONS.

1. *Forensic application of the types M, N, and MN.*

Two laws of the inheritance of the types have been established :—

- (i) The agglutinogens M and N cannot appear in the blood of a child unless present in the blood of one or both parents.

- (ii) A type *M* parent cannot have a type *N* child and a type *N* parent cannot have a type *M* child.

The following table gives the possible and the impossible children :—

Parents.			Children possible.			Children impossible.		
<i>M</i> × <i>M</i>	..		<i>M</i>	<i>MN</i>	<i>N</i>
<i>M</i> × <i>MN</i>	..		<i>M</i>	<i>MN</i>	<i>N</i>
<i>M</i> × <i>N</i>	<i>MN</i>	..	<i>M</i>	..	<i>N</i>
<i>MN</i> × <i>MN</i>	..		<i>M</i>	<i>MN</i>	<i>N</i>	—	—	—
<i>MN</i> × <i>N</i>	<i>MN</i>	<i>N</i>	<i>M</i>
<i>N</i> × <i>N</i>	<i>N</i>	<i>M</i>	<i>MN</i>	..

As has been stated before, for forensic purposes the group is determined before the type.

The genetic basis of the laws is the fact that the factors *M* and *N* are both dominant. The genotypes of the phenotypes *M*, *MN*, and *N* are *MM*, *MN* and *NN*. For further information reference may be made to Wiener (*loc. cit.*). One of us intends to deal with this question in another communication of essentially forensic interest, later.

2. *M* and *N* in transfusion of blood.

It is known that certain donors of blood are better suited for a recipient than others (Snyder, 1929). Are they of the same type as the recipient? Should not the compatibility of both group and type be an important consideration in serious morbid states (like severe anæmias, toxæmias and bacteriæmias) in which a reaction is to be feared?

It is also known that the blood of certain donors causes accidents during a second transfusion. Is the type of the donor in these cases different from that of the recipient, and are the accidents due to a hæmagglutinin produced in the recipients after the first transfusion, against the r.b.c. of a different type?

Again, it is also known that certain donors though of the same group as the recipient are found incompatible on direct matching of bloods. Is this incompatibility caused by a naturally occurring anti-*M* hæmagglutinin (iso-hæmagglutinin)? Such an occurrence is known.

3. Non-specific absorption of anti-*N* substance by *OM* cells.

Such an absorption definitely occurs. It is possible to remove all anti-*N* substance by repeated absorption with *OM* cells.

All the known non-specific reactions between anti-bodies and cells or cell products are either frankly heterologous or are group reactions. To which of these

two categories is the absorption of anti-N substance by OM r.b.c. to be assigned? M and N are too close together in the scheme of things to be heterologous: yet there is no group reaction between them in as much as anti-M substance does not appear to be absorbed by ON r.b.c.

Do mutations appear as new characters arising in the course of variation (as has been held for some time) or do they result from excessive developments of already present potentialities, concurrently with the disappearance of certain other characters (as has been lately thought—Zoogenesis)? Is N a new character or is it merely an excessive development of a potentiality already existing in M? Immunologically it appears to be contained in M but M does not appear to be contained in it. Similar speculation is possible regarding O, A₂, and A. Such speculations are, perhaps, the prerogative of the biologist whose routine keeps him well within 100 per cent biology. The immunologist's interest lies in studying reactions which will give him an insight into the antigenic structure of the haemagglutinogens under discussion. Their antigenic activity is of two distinct orders. The antigenic structure may, of course, not be indicative of an evolutionary process.

4. *M and N in anthropology and genetics.*

Like the statesmen of a certain period in history, who strove for peace and war at the same time, the anthropologists declare against the utility of blood groups in ethnology and yet are grouping the peoples of the earth as assiduously as ever. To the groups now have been added the types. The latter appear to show even a wider range in variation than did the groups, in the obviously distinct ethnic or geographical groups so far studied, and should be more useful.

The wider range in variation should also be more useful to the investigators in genetics. Some of them think that they have caught genes in the actual process of mutation, on certain soils which are virgin with respect to their activities. So far as the types go the whole world is as yet virgin soil and the mutating genes should be caught by the hundred thousand. We have, however, our doubts. In explaining unexpected findings we are more inclined to search for a fault in the technique than to consider possibilities arrived at through mathematical obfuscations. Incidentally, the life of an r.b.c. given by one such obfuscation, once upon a time, was of the order of 30 to 40 days (Evans, 1925); now the life of a transfused corpuscle of the same group but not of the same type as the recipient's red blood cells, detected by immunological means, extends over 100 days (Levine, 1935). It is reasonable to presume that when the donor and the recipient belong to the same group and the same type the life of the corpuscle is prolonged still further. The life of a subject's own r.b.c. must be several months.

SUMMARY.

1. A macroscopic technique of typing blood has been described. Results are read in 5 to 15 minutes, although observation is possible and is recommended for 30 minutes.
2. A method of preparing the anti-M and anti-N fluid has been described. It is an absorption method, needs strict sterility at certain stages and accurate measurements, but is not particularly arduous.

3. Findings in 300 Indians in Calcutta are given : M 42·7 per cent, N 10·7 per cent, and MN 46·7 per cent. Findings in a small series of Europeans in Calcutta and figures from other workers are also given for comparison.

4. Remarks have been made on four associated considerations which are: (i) Forensic application of the types M, N, and MN; (ii) M and N in transfusion of blood; (iii) non-specific absorption of anti-N substance by OM cells; and (iv) M and N anthropology and genetics.

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